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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of	:	Staehler et al.
Application No.	:	10/579,769
Title	:	Highly Parallel Template-Based DNA Synthesizer
Art Unit	:	1637
Examiner	:	Thomas, David C.

Third Party Submission Under 37 CFR 1.99

Hon. Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned submits the following two patents, which are relevant to the above-referenced pending application. This submission complies with all of the requirements of 37 CFR 1.99. Accordingly, the undersigned and respectfully requests that the following two patents are entered in the above-referenced pending application:

U.S. Pat. No. 6,248,521 to Van Ness et al., which published Jun. 19, 2001
U.S. Pat. No. 6,150,102 to Mills, Jr. et al., which published Nov. 21, 2000

Copies of these two patents are submitted herewith.

The total fee of $310.00 set forth in 37 CFR 1.17(i) and (p) is also submitted herewith.

This submission is permitted under 37 CFR 1.99(e) because the patents could not have been submitted to the Office earlier. See MPEP 1134.01(I). Applicants' Amendments filed 07/28/2009 changed the scope of the claims to an extent that could not reasonably have been anticipated by a person reviewing the published application during the period specified in 37 CFR 1.99(e). The extent of the change in the scope of the amended claims is evidenced the Examiner's statement that "further search will be necessary in the next round of prosecution."

08/26/2009 SSANDARA 00000007 10579769
01 FC:1806 180.00 OP

08/26/2009 SSANDARA 00000007 10579769
02 FC:1464 130.00 OP

See the Examiner Interview Summary dated 08/05/2009. Applicants also noted this point in their own Interview Summary, which stated that "Agreement was not reached and the Examiner stated that further search was necessary." See Applicants' Amendment and Response filed 07/28/2009 at page 10.

This submission was served upon the applicant in accordance with 37 CFR 1.248.

Respectfully,

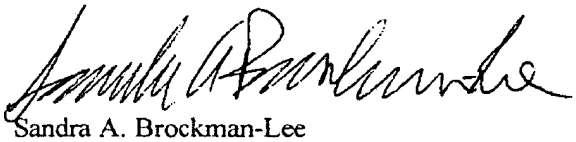
Sandra A. Brockman-Lee
Reg. 44,045

Certificate of Service

I hereby certify on this **21st** day of August 2009, that a true and correct copy of the foregoing "Third Party Submission Under 37 CFR 1.99" was mailed by first-class mail, postage paid, to:

Robert B. Murray, Attorney for the Applicants
ROTHWELL, FIGG, ERNST & MANBECK, P.C.
1425 K STREET, N.W., SUITE 800
WASHINGTON, DC 20005

Respectfully,



Sandra A. Brockman-Lee

US006248521B1

(12) **United States Patent**
Van Ness et al.

(10) **Patent No.: US 6,248,521 B1**
(45) **Date of Patent: Jun. 19, 2001**

(54) **AMPLIFICATION AND OTHER ENZYMATIC REACTIONS PERFORMED ON NUCLEIC ACID ARRAYS**

(75) Inventors: **Jeffrey Van Ness**; **Kristen Moynihan**, both of Seattle; **John C. Tabone**, Bothell, all of WA (US)

(73) Assignee: **Qiagen Genomics, Inc.**, Bothell, WA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/120,501**

(22) Filed: **Jul. 21, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/053,428, filed on Jul. 22, 1997, now abandoned.

(51) **Int. Cl.**[7] **C12Q 1/68**; C12P 19/34; C12P 21/04; B05D 3/04

(52) **U.S. Cl.** **435/6**; 435/91.2; 435/71.1; 427/333

(58) **Field of Search** 435/6, 91.2, 71.1; 427/333

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,020,698	5/1977	D'Autry	73/425.6
4,827,780	5/1989	Sarrine et al.	73/864.21
4,981,783	1/1991	Augenlicht	435/6
5,143,854	9/1992	Pirrung et al.	436/518
5,384,261	1/1995	Winkler et al.	436/518
5,436,327	7/1995	Southern et al.	536/25.34
5,474,796	12/1995	Brennan	427/2.13
5,512,462 *	4/1996	Cheng	435/91.2
5,525,464	6/1996	Drmanac et al.	435/6
5,547,835 *	8/1996	Koster et al.	435/6
5,605,798 *	2/1997	Koster	435/6
5,658,802	8/1997	Hayes et al.	436/518
5,709,668	1/1998	Wacks	604/232
5,741,554	4/1998	Tisone	427/424
5,741,637 *	4/1998	Rueger et al.	435/6
5,744,305 *	4/1998	Fodor et al.	435/6
5,770,151	6/1998	Roach et al.	422/63
5,770,367	6/1998	Southern et al.	435/6
5,800,992	9/1998	Fodor et al.	435/6
5,807,522	9/1998	Brown et al.	422/50
5,846,710 *	12/1998	Bajaj	435/6
5,919,523 *	7/1999	Sundberg et al.	427/333
6,101,946	8/2000	Martinsky	101/494

FOREIGN PATENT DOCUMENTS

WO 90/13666	11/1990	(WO) .
WO 91/07505	5/1991	(WO) .
WO 93/19207	9/1993	(WO) .
WO 94/29484	12/1994	(WO) .
WO 95/20679	8/1995	(WO) .
WO 95/33073	12/1995	(WO) .
WO 96/04404	2/1996	(WO) .
WO 96/31622	10/1996	(WO) .

OTHER PUBLICATIONS

Lu et al., "Use of glycerol for enhanced efficiency and specificity of PCR amplification", Trends in Genetics, vol. 9(9), p. 297, Sep. 1993.*

Blanchard et al., (1996), "High-density oligonucleoyide arrays," *Biosens. Bioelectron. 11*: 687–690.

Chee et al., (1996), "Accessing genetic information with high-density DNA arrays," *Science 274*: 610–614.

Chu et al., (1998), "The transcriptional program of sporulation in budding yeast," *Science 282*, 699–705.

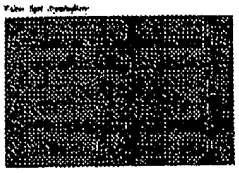
(List continued on next page.)

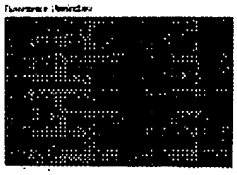
Primary Examiner—Jeffrey Fredman
Assistant Examiner—Arun Chakrabarti
(74) *Attorney, Agent, or Firm*—Seed Intellectual Property Law Group PLLC

(57) **ABSTRACT**

The present invention provide methods and an apparatus for performing amplification and other enzymatic reactions on nucleic acid molecules that have been printed onto a solid substrate, such as a silicon wafer or glass slide.

41 Claims, 5 Drawing Sheets





OTHER PUBLICATIONS

Cronin et al. (1996), "Cystic Fibrosis Mutation Detection by Hybridization to Light-Generated DNA Probe Arrays," *Human Mutation 7*: 244–255.

DeRisi et al. (1996), "Use of a cDNA microarray to analyze gene expression patterns in human cancer," *Nat Genet 14*: 457–460.

DeRisi et al., (1997), "Exploring the metabolic and genetic control of gene expression on a genomic scale," *Science 278*: 680–686.

de Saizieu et al., (1998), "Bacterial transcript imaging by hybridization of total RNA to oligonucleotide arrays," *Nature Biotech. 16*: 45–48.

Drmanac et al., (1998), "Accurate sequencing by hybridization for DNA diagnostics and individual genomics," *Nature Biotech. 16*: 54–58.

Fodor et al., (1991), "Light-directed, spatially addressable parallel chemical synthesis," *Science 251*: 767–773.

Hacia et al., (1996), "Detection of heterozygous mutations in BRCA1 using high density oligonucleotide arrays and two-colour fluorescence analysis," *Nature Genet. 14*: 441–447.

Heller et al., (1997), "Discovery and analysis of inflammatory disease-related genes using cDNA microarrays," *Proc Natl Acad Sci USA. 94*: 2150–2155.

Khrapko et al., (1991), "Hybridization of DNA with oligonucleotides immobilized in gel: a convenient method for detecting single base substitutions," *Molecular Biology 25*: 581–591.

Kozal et al., (1996), "Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays," *Nature Med. 2*: 753–759.

Lashkari et al., (1997), "Yeast microarrays for genome wide parallel genetic and gene expression analysis," *Proc. Natl. Acad. Sci. USA 94*: 13057–13062.

Lemieux et al., (1998) "Overview of DNA Chip Technology," *Molecular Breeding 4*: 277–289.

Lockhart et al., (1996), "Expression Monitoring by Hybridization to High-Density Oligonucleotide Arrays," *Nature Biotechnology 14*: 1675–1680.

Maier et al. (1994), "Application of robotic technology to automated sequence fingerprint analysis by oligonucleotide hybridisation," Summary.

Pease et al., (1994), "Light-generated oligonucleotide arrays for rapid DNA sequence analysis," *Proc. Natl. Acad. Sci. USA 91*: 5022–5026.

Sapolsky and Lipshutz, (1996), "Mapping Genomic Library Clones Using Oligonucleotide Arrays," *Genomics 33*: 445–456.

Schena et al., (1995), "Quantitative monitoring of gene expression patterns with a complementary DNA microarray," *Science 270*:467–470.

Schena, M., (1996), "Genome Analysis with Gene Expression Microarrays," BioEssays 18: 427–431.

Schena et al., (1996), "Parallel human genome analysis: microarray-based expression monitoring of 1000 gene," *Proc Natl Acad Sci USA 93*: 10614–10619.

Schena et al., (1998), "Microarrays: Biotechnology's discovery platform for functional genomics," *Trends Biotech. 16*: 301–306.

Schena and Davis, (1998), "Parallel Analysis with Biological Chips. in PCR Methods Manual," Academic Press (San Diego), in press.

Shalon et al., (1996), "A DNA micro-array system for analyzing complex DNA samples using two-color fluorescent probe hybridization," *Genome Research 6*: 639–645.

Shoemaker et al., (1996), "Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy," *Nature Genetics 14*: 450–456.

Wodicka et al., (1997), "Genome-wide expression monitoring in *Saccharomyces cerevisiae*," *Nature Biotech. 15*: 1359–1367.

Yershov et al. (1996), "DNA analysis and diagnostics on oligonucleotide microchips," *Proc. Natl. Acad. Sci. USA 93*: 4913–4918.

cmgm.stanford.edu/pbrown/mguide, Sep. 12, 2000 and cmgm.stanford.edu/pbrown/mguide/tips. Sep. 12, 2000.

* cited by examiner

Visible light illumination:

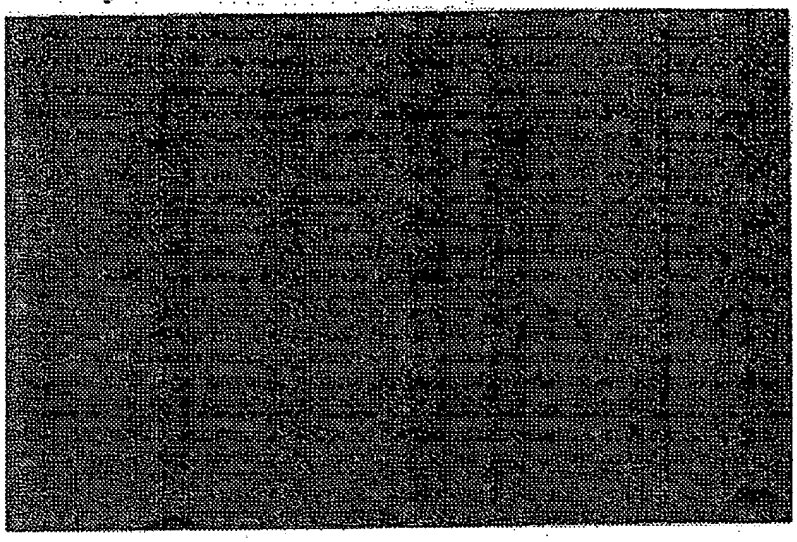


Fig. 1A

Fluorescence illumination:

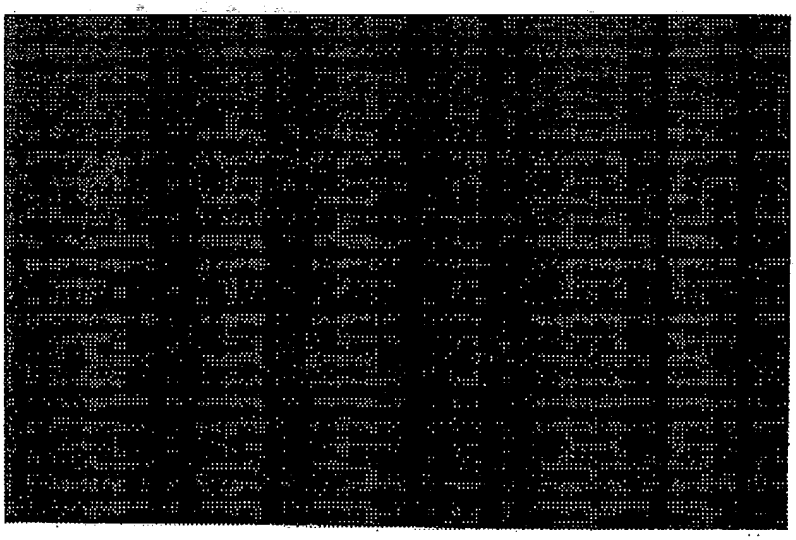


Fig. 1B

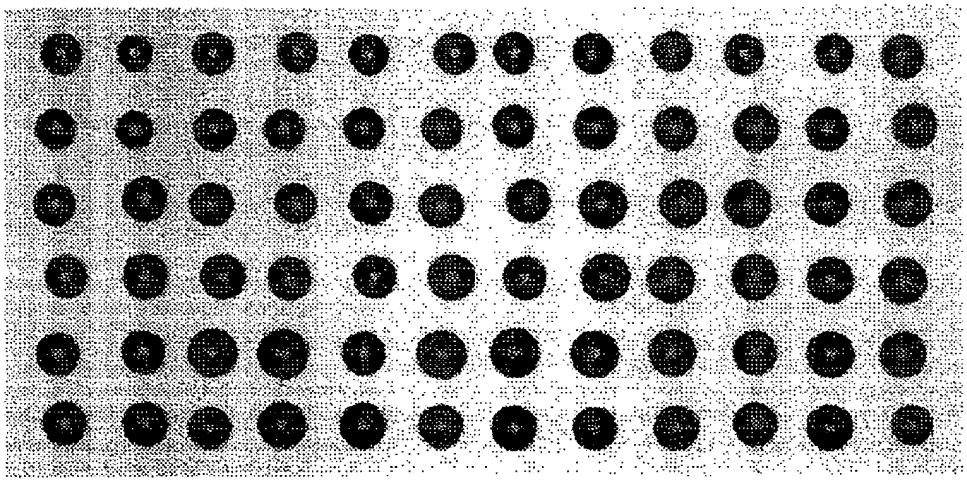


Fig. 2

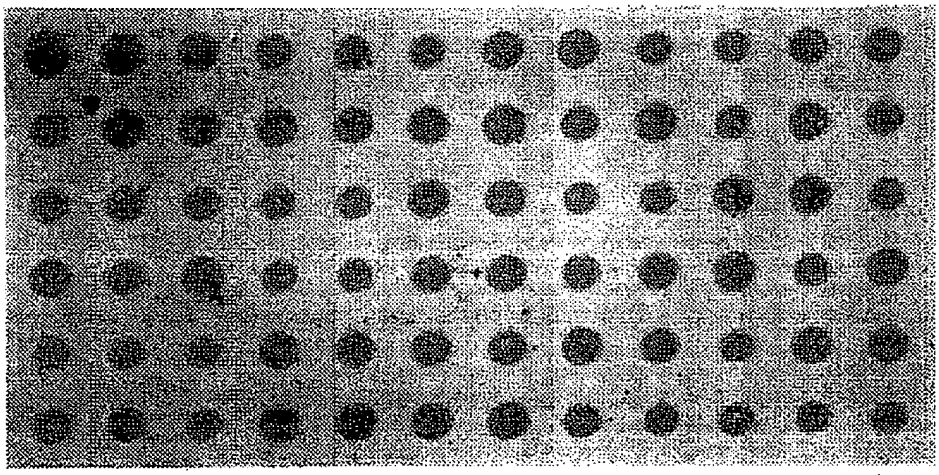


Fig. 3

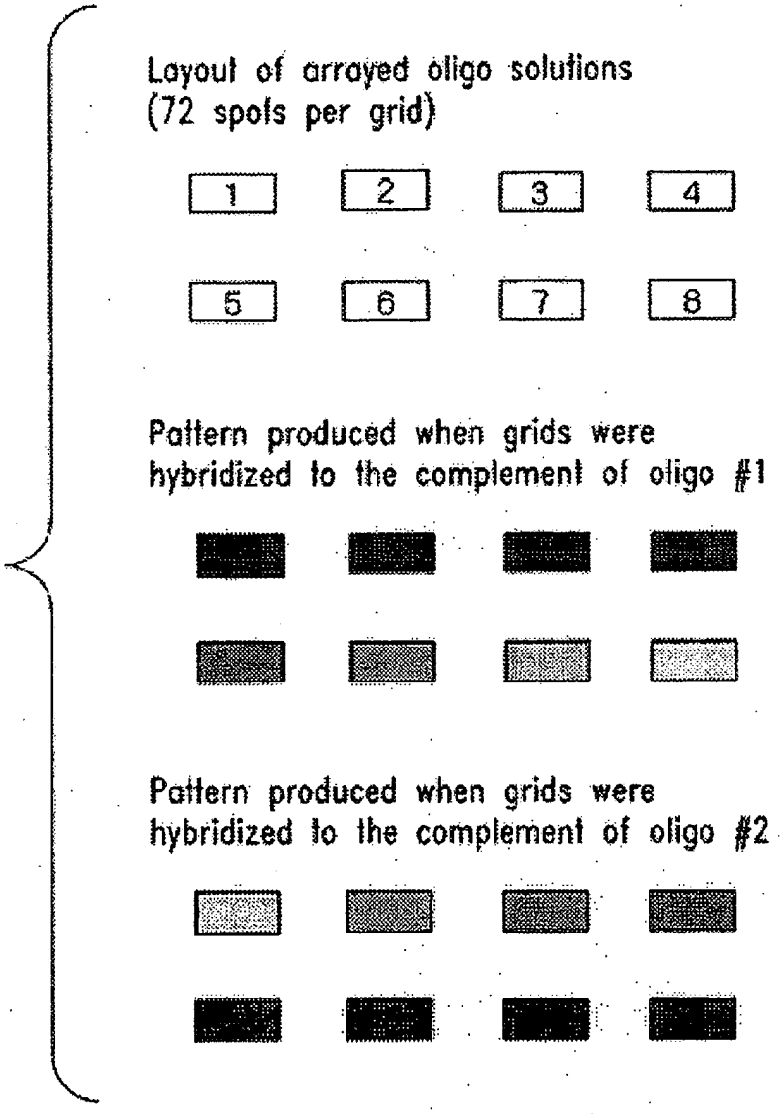

Layout of arrayed oligo solutions
(72 spots per grid)
1
2
3
4
5
6
7
8
Pattern produced when grids were
hybridized to the complement of oligo #1
Pattern produced when grids were
hybridized to the complement of oligo #2

Fig. 4

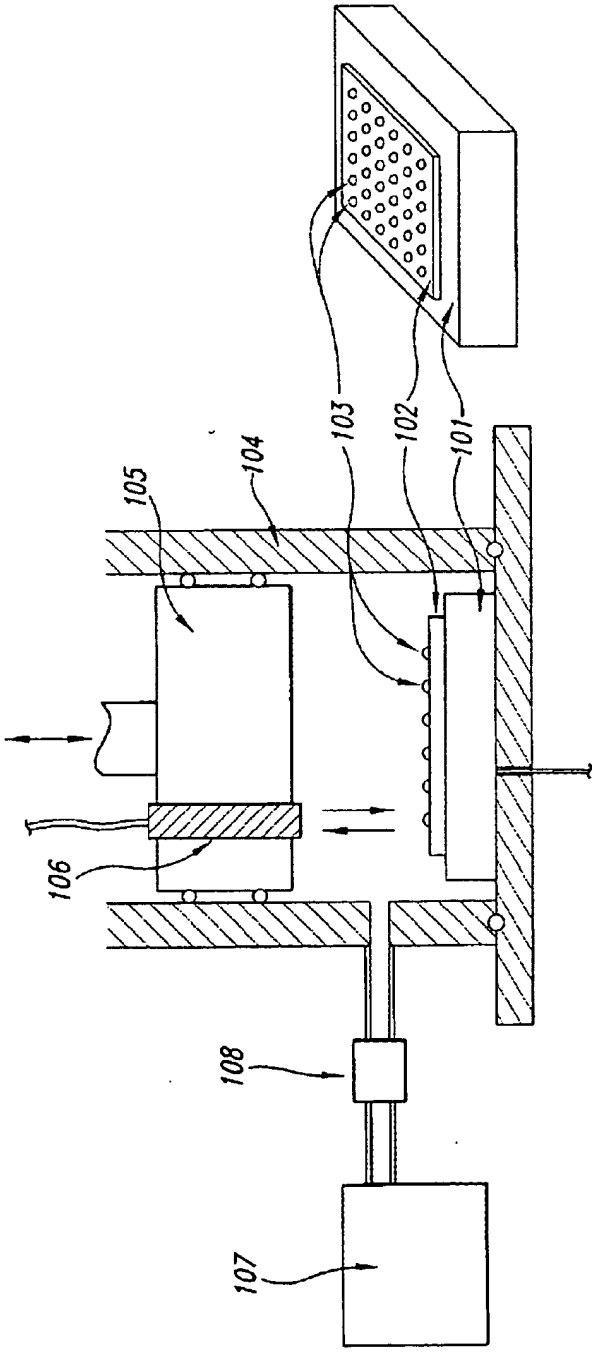


Fig. 5

AMPLIFICATION AND OTHER ENZYMATIC REACTIONS PERFORMED ON NUCLEIC ACID ARRAYS

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims the benefit of U.S. Provisional Patent Application No. 60/053,428, filed Jul. 22, 1997, now abandoned.

TECHNICAL FIELD

This invention relates generally to enzymatic reactions performed on nucleic acids that are arrayed on a solid substrate, and in particular, to amplification of nucleic acids that are arrayed.

BACKGROUND OF THE INVENTION

Replicate arrays of biological agents have been used to facilitate parallel testing of many samples. For example, sterile velvet cloths and a piston-ring apparatus has long been used to make replicates of bacterial and yeast colonies to agar plates each containing a different growth medium, as a means of rapidly screening a large number of independent colonies for different growth phenotypes (Lederberg and Lederberg, *J. Bacteriol.* 63 :399, 1952). Likewise, 96-well microtiter plates are used to organize and store in an easily accessed fashion large numbers of e.g. cell lines, virus isolates representing recombinant DNA libraries, or monoclonal antibody cell lines.

The advent of large scale genomic projects and the increasing use of molecular diagnostics has necessitated the development of large volume throughput methods for screening nucleic acids. Recently, methods have been developed to synthesize large arrays of short oligodeoxynucleotides (ODNs) bound to a glass or silicon surface that represent all, or a subset of all, possible nucleotide sequences (Maskos and Southern, *Nucl. Acids Res.* 20: 1675, 1992). These ODN arrays have been made used to perform DNA sequence analysis by hybridization (Southern et al., *Genomics* 13: 1008, 1992; Drmanac et al., *Science* 260: 1649, 1993), determine expression profiles, screen for mutations and the like. In all these uses, the ODNs are covalently attached to the surface of the substrate. However, some useful screening techniques and assays are not readily adaptable to a format in which ODNs are immobilized.

In particular, amplification of nucleic acids, notably the polymerase chain reaction (PCR) and its many variations, has found wide application to many different many biological problems and is not easily moved to a format where the ODNs are immobilized. In its standard format, PCR has two major limitations to its commercial utilization: the cost of reagents and the ability to automate the process. Reagent costs, especially DNA polymerase, can be lowered if the total volume of each reaction is decreased. An accurate and reliable means to array small volumes of reagents using a robotically controlled pin tool would miniaturize the reactions. Additional hurdles to moving amplification to an array format include preventing evaporation during heating and cooling cycles and preventing spreading and merging of the reactions on the array.

The present invention discloses methods and compositions for performing amplification and other enzymatic reactions in an array format without the need to immobilize the components, and further provides other related advantages.

SUMMARY OF THE INVENTION

Within one aspect of the present invention, methods of amplifying nucleic acid molecules from a template are provided comprising (a) mixing single-stranded nucleic acid templates on a solid substrate with a solution comprising an oligonucleotide primer that hybridizes to the templates and a DNA polymerase, wherein the mixing occurs in discrete areas on the substrate, and wherein the solution remains in the discrete areas; (b) synthesizing a complementary strand to the template to form a duplex; (c) denaturing the duplex; and (d) synthesizing complementary strands to the template, therefrom amplifying nucleic acid molecules; wherein mixing, synthesizing, and denaturing are conducted at dew point. The solid substrate may be a silicon wafer or glass slide. The templates may be covalently attached to the solid substrate or deposited on the surface of the substrate. The template may be uniformly applied to the entire array prior to mixing or applied individually to each discrete area on the substrate. When applied individually, preferably the applying is performed using spring probes. In a most preferred embodiment, an apparatus is used to control the dew point.

Within a related aspect, the method of amplifying uses a first oligonucleotide primer that hybridizes to the templates, a second oligonucleotide primer that hybridizes to a complementary strand of the template, and after sythesizing, denaturing the duplex; and synthesizing complementary strands to the template and the complementary strand of the template, therefrom amplifying nucleic acid molecules.

In preferred embodiments, the denaturing and synthesizing steps are performed multiple times. In other preferred embodiments, the solution contains a compound that confers viscosity, such as glycerol or a sugar. In other preferred embodiments, the DNA polymerase is a thermostable polymerase and synthesis and denaturation are performed at different temperatures.

In yet other preferred embodiments, the method further comprises detecting the duplexes. Most preferably, the oligonucleotide primers are labeled with a tag that is detectable by non-fluorescent spectrometry or potentiometry, and preferably by mass spectrometry, infrared spectrometry, ultraviolet spectrometry, or poteniostatic amperometry.

In another aspect, a method of synthesizing a nucleic acid molecule from a template is provided, comprising (a) mixing single-stranded nucleic acid templates on a solid substrate with a solution comprising an oligonucleotide primer that hybridizes to the templates and a DNA polymerase, wherein the mixing occurs in a discrete area of an array, and wherein the solution remains in discrete areas; and (b) synthesizing a complementary strand to the template to form a duplex, wherein mixing and synthesis are performed at dew point, wherein dew point is maintained or achieved by an apparatus, comprising: a container capable of being pressurized; a heating device; a means for generating pressure; and a means for generating saturated steam; wherein the heating device, pressure generating means, and steam generating means are controllable.

In yet another aspect, a method of detecting a single base alteration in a nucleic acid molecule, is provided comprising (a) mixing single-stranded nucleic acid molecules on a solid substrate with a solution comprising a first and a second oligonucleotides that hybridize to the nucleic acid molecules and a DNA ligase, wherein the mixing occurs in a discrete area of an array, and wherein the solution remains in the discrete areas; and (b) detecting a ligation product; wherein the first and second oligonucleotides will not ligate when there is a single base alteration at the junction base on the nucleic acid molecule, wherein mixing is performed at dew point.

In yet another aspect, a method of performing single nucleotide extension assay is provided, comprising (a) mixing oligonucleotides on a solid substrate with a solution comprising single-stranded nucleic acid molecules that hybridize to the oligonucleotides, a single nucleotide, and a DNA polymerase, wherein the mixing occurs in discrete areas of the substrate, and wherein the solution remains in discrete areas; and (b) detecting an extension product of the oligonucleotide; wherein the oligonucleotide will be extended only when the single nucleotide is complementary to the nucleotide adjacent to the hybridized oligonucleotide, wherein mixing is performed at dew point.

In other aspects, the invention provides a kit for genotyping, comprising a solid substrate containing an array of labeled oligonucleotide primer pairs. In preferred embodiments, the kit further comprises nucleic acid templates and a viscous solution.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (e.g., plasmids, etc.), and are therefore incorporated by reference in their entirety.

The methods and kits of the present invention may include tagged biomolecules, for example, oligonucleotides covalently bonded to cleavable tags. Exemplary tagged biomolecules, and assays which may use the same, are described in U.S. patent application Ser. Nos. 08/786,835; 08/786,834 and 08/787,521, each filed on Jan. 22, 1997, as well as in three U.S. continuation-in-part patent applications having Application Ser. Nos. 08/898,180; 08/898,564; and 08/898,501, each filed Jul. 22, 1997 and PCT International Publication Nos. WO 97/27331; WO 97/27325; and WO 97/27327. These six U.S. Patent Applications and three PCT International Publications are each hereby fully incorporated herein by reference in their entireties.

The methods and kits of the present invention may be used in conjunction with arrays that contain more than one oligonucleotide sequence within an element (or "first region"). Biomolecule arrays containing more than one oligonucleotide sequence within an element, and uses thereof, are described in our U.S. patent application Ser. No. 09/120,688 titled "Multiple Functionalities Within An Array Element And Uses Thereof" filed concurrently herewith, which claims the priority benefit of U.S. Provisional Patent Application No. 60/053,436 filed Jul. 22, 1997, both of which are hereby fully incorporated herein by reference in their entireties.

Biomolecule arrays that may be used in conjunction with the methods and kits of the present invention may be prepared according to techniques disclosed in our U.S. patent application Ser. No. 09/120,689 titled "Apparatus and Methods For Arraying Solution Onto A Solid Support" filed concurrently herewith, which claims the priority benefit of U.S. Provisional Patent Application No. 60/053,435 filed Jul. 22, 1997, both of which are hereby fully incorporated herein by reference in their entireties.

Biomolecule arrays that may be used in conjunction with the methods and kits of the present invention may be prepared according to techniques as disclosed in our U.S. patent application Ser. No. 09/120,386 titled "Polyethylenimine-Based Biomolecule Arrays" filed concurrently herewith, which claims the priority benefit of U.S. Provisional Patent Application No. 60/053,352 filed Jul. 22, 1997, both being fully incorporated herein by reference in their entireties.

Computer systems and methods for correlating data, as disclosed in U.S. patent application Ser. No. 09/120,686 titled "Computer Method and System For Correlating Data" filed concurrently herewith, which claims the priority benefit of U.S. Provisional Patent Application No. 60/053,429 filed Jul. 22, 1997 (both being fully incorporated herein by reference in their entireties) may be used in combination with the amplification and other enzymatic reactions performed on nucleic acid arrays as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows photomicrographs of arrayed microspheres taken under visible light illumination (top panel) and fluorescence illumination (bottom panel).

FIG. 2 shows a CCD camera image of an array produced by a robot using the methodology of the invention, where the domains are approximately 100–150 microns in average diameter with 200 micron center to center spacing between spots. The standard deviation of spot diameter is approximately 15%.

FIG. 3 shows an array of microspots prepared according to the invention and developed using Vector Blue (Vector Laboratories, Burlingame, Calif.) and imaged with a CCD camera and microscope.

FIG. 4 is an illustration showing how two different oligonucleotides, both present within a single array element, may be identified and partially quantified according to the present invention.

FIG. 5 is an illustration of an apparatus that controls dew point.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention provides methods and apparatus for amplifying nucleic acid molecules from a template as well as methods and apparatus for performing enzymatic assays on nucleic acid molecules. These methods are generally performed on an array of nucleic acid molecules made as described herein. In the present invention, these methods are performed in an apparatus that controls dew point.

I. Application of Templates to Solid Substrate

A. Substrate preparation

A substrate for arrays is prepared from a suitable material. The substrate is preferably rigid and preferably has a surface that is substantially flat. In some embodiments, the surface may have raised rigids to delineate regions. Typical substrates are silicon wafers and borosilicate slides (e.g., microscope glass slides), although other materials known in the art may be substituted. An example of a particularly useful solid support is a silicon wafer that is typically used in the electronics industry in the construction of semiconductors. The wafers are highly polished and reflective on one side and can be easily coated with various linkers, such as poly(ethyleneimine) using silane chemistry. Wafers are commercially available from companies such as WaferNet, San Jose, Calif.

Nucleic acid molecules or other biopolymers, such as peptides, may either be synthesized in situ, i.e., on the solid substrate, or synthesized elsewhere and applied to the substrate. Alternatively, substrates with oligonucleotides already present in arrays can be purchased (e.g., Affymetrix, Palo Alto, Calif). Many suitable methods for synthesizing nucleic acids on a solid substrate, such as a silicon wafer, are readily available. These methods rely on standard protocols,

such as phosphoramidite chemistry, to synthesize an oligonucleotide. Nucleic acids and peptides may also be synthesized in an automated fashion using a commercially available machine. A preferred method is to prepare the nucleic acid molecules and apply them to the substrate. In certain embodiments, the molecules are covalently attached to the substrate. In preferred embodiments, the nucleic acids are deposited on the solid substrate and are not covalently attached.

In certain embodiments, the surface of the substrate is prepared for the oligonucleotides. The surface may be prepared by, for example, coating with a chemical that increases or decreases the hydrophobicity or coating with a chemical that allows covalent linkage of the nucleic acid molecules or other polymeric sequences. Some chemical coatings may both alter the hydrophobicity and allow covalent linkage. Hydrophobicity on a solid substrate may readily be increased by silane treatment or other treatments known in the art. A chemical that allows covalent linkage is generally referred to as a linker. These linker molecules adhere to the surface of the substrate and comprise a functional group that reacts with biomolecules. Many such linkers are readily available. For example, solid supports are modified with photolabile-protected hydroxyl groups (see, U.S. Pat. Nos. 5,412,087; 5,571,639; 5,593,839), alkoxy or aliphatic derivatized hydroxyl groups (U.S. Pat. No. 5,436, 327), or other chemicals (see e.g., U.S. Pat. No. 5,445,934; EP Patent No. EP-B1-0,373,203; U.S. Pat. No. 5,474,796; U.S. Pat. No. 5,202,231)

A preferred coating that both decreases hydrophobicity and provides linkers is poly(ethyleneimine). In addition, poly(ethyleneimine) (PEI) coated solid substrates also have the added benefit of long shelf life stability. The coating of silicon wafers and glass slides with polymers such as poly(ethyleneimine) can be performed in-house or through companies such as Cel Associates (Houston, Tex.). Glass slides can also be coated with a reflective material or coated with PEI using silane chemistry. The PEI coating permits the covalent attachment of single or double stranded oligonucleeotides, single or double stranded long DNA molecules or fragments or any other amine-containing biomolecules to the solid support. Oligonucleotides may be covalently attached at the 5' using a hexylamine modification, which places a primary amine at the 5'-end of the oligonucleotide. The 5'-amine on the oligonucleotide may then be reacted with a cross-linker, such that the oligonucleotide is covalently attached to the polymer coating on the solid support.

Any nucleic acid type can be covalently attached to a PEI coated surface as long as the nucleic acid contains a primary amine. Amplified products (e.g, by PCR) may be modified to contain a primary amine by using 5'-hexylamine-coujugated primers. Amine groups may be introducted into amplified products and other nucleic acid duplexes by nick translation using allyl-dUTP (Sigma, St. Louis, Mo.). As well, amines may be introduced into nucleic acids by polymerases, such as terminal transferase, or by ligation of short amine-containing oligonucleotides. Other suitable methods known in the art may be substituted.

Cross linkers suitable for amine groups are generally commercially available (see, e.g., Pierce, Rockford, Ill.). A typical cross-linker is trichlorotriazine (cyanuric chloride) (Van Ness et al., *Nucleic Acids Res.* 19: 3345–3350, 1991). Briefly, an excess of cyanuric chloride is added to the oligonucleotide solution (e.g., a 10 to 1000-fold molar excess of cyanuric chloride over amines) at a typical oligonucleotide concentration of 0.01 to 1 μg/ml, and preferably about 0.1 μg/ml. The reaction is buffered using common buffers such as sodium phosphate, sodium borate, sodium carbonate, or Tris HCL at a pH range from 7.0 to 9.0. The preferred buffer is freshly prepared 0.2 M NaBorate at pH 8.3 to pH 8.5. Ten μl of 15 mg/ml solution of cyanuric chloride is added and allowed to react with constant agitation from 1 to 12 hours and preferably approximately 1 hour. Reaction temperature may range from 20 to 50° C. with the preferred reaction temperature at 25° C. (or ambient temperature).

When cyanuric chloride is used as a cross linker, there is no need to remove the excess crosslinker prior to printing the nucleic acids on a solid substrate. Excess cyanuric chloride in the reaction mixture does not interfer or compete with the covalent attachment of the nucleic acid or oligonucleotides to the PEI coated solid support, because of an excess of amines on the solid support over the number of cyanuric chloride molecules. In a preferred embodiment, cross-linked oligonucleotides are not purified prior to the printing step.

If the nucleic acids or other amine-containing polymers are to be covalently attached, the activated polymers are allowed to react with the solid support for 1 to 20 hours at 20 to 50° C. and preferably for 1 hour at 25° C. The free amines on the solid support are then capped to prevent non-specific attachment of other nucleic acids. Capping is accomplished by reacting the solid support with 0.1 to 2.0 M succinic anhydride, and preferably 1.0 M succinic anhydride in 70% m-pyrol and 0.1 M NaBorate, for 15 minutes to 4 hours with a preferred reaction time of 30 minutes at 25° C. The solid support is then incubated in a 0.1 to 10.0 M NaBorate, pH 7 to pH 9 (preferably 0.1 M NaBorate pH 8.3) solution containing 0.1 to 5 M glycine (preferably 0.2 M glycine) and then washed with detergent-containing solution. This "caps" any dichloro-triazine that may be covalently bound to the PEI surface. Preferrably, the solid support is further heated to 95° C. in 0.01 M NaCl, 0.05 M EDTA and 10 mM Tris pH 8.0 for 5 minutes to remove any non-covalently attached nucleic acids. In the case where double stranded nucleic acids are printed onto a solid substrate, this step also converts (denatures) the double strand to a single strand form.

B. Methods of applying nucleic acid molecules to solid substrates

Oligonucleotides, nucleic acid molecules or other biopolymers are "printed" (delivered or applied) on a solid substrate. In preferred embodiments, the polymers are applied in a regular pattern or array. In other preferred embodiments, the polymers are applied to the entire area of the solid substrate and allowed to dry, after which additional polymers, buffers, enzymes and the like are applied in an array pattern. The polymers may be applied to the substrate in a buffered salt solution without detergents, such as 10 mM Tris, 50 mM NaCl, and 5 mM EDTA, using a pipettor, nylon roller, stamps, or the like.

A variety of printing methods are available for applying nucleic acids, such as oligonucleotides or DNA fragments, to a solid substrate in an array pattern. As a general guideline, the delivery mechanism must be capable of positioning very small amounts of liquids (e.g., nanoliters) in small regions (e.g., 10–200 μm diameter dots) where the regions are very close to one another (e.g., 25–500 μm center to center distance). Preferably the printing technique is amenable to automation. One such technique is ink-jet printing using multiple heads. Very fine pipettes may also be used. A preferred means of printing is using spring probes as described herein.

Sample pick-up, transfer and micro-droplet deposition is greatly enhanced when using a liquid transfer device that has

a hydrophilic surface, especially when that device is a modified spring probe. Spring probes are made hydrophilic through the use of chemical agents acting to modify the surface of the probe or through coating the probe with a hydrophilic substance. In a preferred method, the tip of the spring probe is soaked in a 25–200 mM solution of 1,4-dithiothreitol, 0.1 M sodium borate for 15 min to 2 hrs. Dithiothreitol reacts with gold surfaces through a thiol-gold coordination, which essentially hydroxylates the surface, making it hydrophilic.

The hydrophilic surface promotes an even coating of sample when the spring probe is dipped in solution. The fluted probe becomes evenly and consistently loaded with liquid drawn to the probe surface by its hydrophilic nature. Solutions with viscosity enhancing chemicals, such as glycerol, provide especially improved handling capabilities using hydrophilic surfaces. With these solutions, the glycerol adheres to the probe even as it pulled from the source of liquid. As a sample is transferred from its source to a solid support, the hydrophilic surface of the probe continues to benefit liquid handling by retaining the sample being transferred and inhibiting the sample from randomly dripping or running during transport. When a sample bearing spring probe comes into contact with a solid support, the sample is deposited from the tip of the spring probe onto the surface of the solid support, especially in the case of a sample containing a viscosity enhancing solution. The size of the areas spotted generally range from 10–200 μm with a typical center to center distance of 25–500 μm.

Briefly, in a typical procedure, a solution of the nucleic acid is uniformly mixed in 57% glycerol and then printed onto the solid support. Within the context of this invention, the biopolymers may be either nucleic acid molecules or protein molecules. When nucleic acids are used, they may comprise single or double stranded DNA, single or double stranded RNA, oligonucleotides, hybrid DNA-RNA molecules or duplexes, PNA nucleic acids with a protein backbone and the like.

II. Reaction Components and Conditions

As noted above, the present invention provides methods for amplifying nucleic acids on a solid substrate as well as other enzymatic reactions. As noted above, the nucleic acids may be covalently attached to the surface of the substrate or may be deposited on the substrate without attachment. Typically, the template for amplification is printed first and reagents necessary for amplification or other enzymatic reactions are subsequently added.

A. Reagents, buffers, cofactors, etc.

Each area of the array that undergoes a reaction has in addition to the template nucleic acids, the appropriate enzyme, and any other required components, including, but not limited to, oligonucleotide primers, nucleotides, buffers, cofactors, and the like. For example, an amplification reaction includes template, DNA polymerase, nucleotides (e.g., dATP, dCTP, dGTP, dTTP), oligonucleotide primers, and buffer containing a divalent cation, usually Mg^{++}.

Amplification reactions are based on primer extensions (e.g., polymerase chain reaction, see U.S. Pat. Nos. 4,683,195; 4,683,202; and 4,800,159, cycling probe technology, NASBA), ligation (LCR, ligation chain reaction), RNA amplification (see Lizardi et al., *Bio/Technology* 6:1197, 1988; Kramer et al., *Nature* 339:401, 1989; Lomeli et al., *Clin. Chem.* 35:1826, 1989; U.S. Pat. No. 3,786,600), differential display (Liang and Pardee, *Science*, 257: 967–971, 1992; Liang, et al., *Nucl. Acids Res.*, 22:5763–5757, 1994), and the like. Preferably, the amplification method is polymerase chain reaction with a thermostable DNA polymerase, such as Taq DNA polymerase, $Vent_R$®DNA polymerase, $Vent_R$®(exo-) DNA polymerase, Pfu DNA polymerase, and the like. For these enzymes, optimal buffers and divalent cations are well known. Oligonucleotide primers are preferably average G+C content and with non-pairing 3' ends. Oligonucleotide sequence will also depend in part upon the region to be amplified. Conditions and considerations for oligo design, buffer concentrations and cation concentrations are well known (see, e.g., Ausubel et al. *Current Protocols in Molecular Biology*, Greene Publishing, 1995; Innis et al., PCR *Protocols: A Guide to Methods and Applications*, Academic Press, 1990; Sambrook et al. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1987). The nucleotides are generally the four deoxynucleotides, dATP, dCTP, dGTP, and dTTP, but may also include derivatives or rare bases.

Other enzymatic reactions within the context of this invention include synthesis of a nucleic acid molecule from a template, oligonucleotide ligation assay to detect a single base alteration in a nucleic acid molecule and a single nucleotide extension assay. For each of these methods, suitable conditions are well known.

In addition, the reactions may contain other chemicals or components. (see U.S. application Ser. Nos. 08/719,132 and 60/026,621, and International Publication Number WO 98/13527 which claims priority to these two U.S. Applications, all of which are incorporated herein in their entireties). For example, a hybotrope may be added to improve annealing of an oligonucleotide primer to template. A hybotrope refers to any chemical that can increase the enthalpy of a nucleic acid duplex by 20% or more when referenced to a standard salt solution (i.e., 0.165 M NaCl). A chemical exhibits hybotropic properties when, as a solution an 18 bp oligonucleotide duplex that is 50% G+C has a helical-coil transition (HCT) of 15° C. or less. HCT is the difference between the temperatures at which 80% and 20% of the duplex is single-stranded. The temperature for annealing is then chosen to be the discrimination temperature, which is a temperature at which a hybridization reaction is performed that allows detectable discrimination between a mismatched duplex and a perfectly matched duplex. A range of temperatures satisfy criteria of a discrimination temperature.

In a preferred embodiment, the reactions are performed in a viscous solution. Such a solution preferably raises the dew point (i.e., lowers vapor pressure), has a high surface tension, and improves printing ability. The viscous solution must not substantially inhibit enzymatic activity. Preferably, enzyme activity is inhibited less than 1 to 20%. Suitable compounds to increase viscosity include glycerol and sugars. Preferably, glycerol is present at 20–100% and more preferably at 20–70%. Other suitable compounds may be identified by (a) determining enzyme activity in the presence of the compound, and (b) forming drops on a solid substrate, incubating at the reaction temperature, and observing that discrete drops (areas) remain. In general, the more hydrophobic the substrate surface, the lower the viscosity solution, and the more hydrophilic the substrate surface, the higher the viscosity solution.

B. Apparatus to maintain dew point

As noted above, the reactions are performed at dew point. Dew point, as used herein, refers to a temperature range where the droplet size does not change significantly. As described herein, an apparatus capable of controlling temperature, pressure, and water content may be used to maintain dew point.

As such, the reactions are conducted under pressure with a defined water content level that prevents the evaporation of water from the microdroplet. These conditions are achieved when there is an equilibrium state between the rate of evaporation of water from the microdroplet and the rate of condensation of water onto the microdroplet from the moist air overlying the substrate arrays. When this equilibrium is realized, the air is said to be saturated with respect to the planar surface of the array. The pressure (Ps) exerted by the water vapor is the saturation vapor pressure that must be maintained at any given temperature during the reaction. The magnitudes of the saturation vapor pressures depend only on temperature and increase rapidly with increasing temperature. That is, thermocycling amplification are conducted at essentially the dew points for all the temperatures achieved. For example, at 0° C., the absolute pressure of saturated steam is 0.0885 psi whereas at 100° C., the absolute pressure of saturated steam is 17.186 psi. Therefore, an apparatus should have the ability to maintain the dew point during all the temperature cycling that occurs during amplification or other enzymatic reaction. Essentially, saturated clean steam will be present in the "chamber." The apparatus is typically composed of a pressure chamber that contains the solid support, a controllable heating device, a means for generating pressure, and a means for generating saturated steam. All parameters are preferably controllable by computer. In other embodiments, the apparatus is a chamber with a means for generating pressure, a means for generating saturated steam and a seal, such that the chamber is sealed onto a controllable heating and cooling block (such as those commercially available). This modular apparatus is designed to fit formats of heating and cooling blocks of various sizes, e.g., from a 96-well plate size to a microscope slide.

In a preferred embodiment, and in reference to FIG. 5, the invention provides a heating and cooling block **101** on which sits a glass cover slip **102**, which contains the discrete areas of sample drops **103**. The block is encased in an airtight cover **104** that forms a chamber, which has a piston **105** to adjust the internal pressure, a sensor **106** to measure dew point, and a source of water vapor **107**.

In a preferred embodiment of the invention, the apparatus for opening the chamber, the temperature of the block, the position of the piston, and the valve are all under computer control. In one embodiment of the invention, the sensor **(106)** is a CCD camera and a light source behind a transparent section of the piston. In this embodiment, the size of one or more of the drops is continuously measured by imaging the drop(s) and comparing the drop image(s) to the image of a reference spot. The dew point is estimated by monitoring the drop size, and the pressure is adjusted to maintain the drops at their original size. The pressure is controlled by controlling the position of the piston. In another embodiment of the invention, the pressure is monitored using conventional sensors. In this embodiment, the pressure is varied to preset values which are based on the sample temperature and sample composition to fall within the predicted dew point range.

In a preferred embodiment of the invention, the source of water vapor **(107)** consists of source of dry gas which is passed through a water-saturated filter held at a constant temperature. The gas flowing out of the vapor source is saturated with water and at a controlled temperature. This gas is used to flush the chamber before the chamber is sealed, and serves to ensure that the composition of the atmosphere in the chamber is consistent and require evaporation from the samples to reach equilibrium.

III. Detection of Reaction Products

Reaction products may be detected by a variety of methods. Preferably, one of the reaction components is labeled. In amplification reactions, the oligonucleotide primers or the nucleotides are conveniently labeled. Preferably, the primers contain a label. In single nucleotide extension assay, the added nucleotide is generally labeled, in oligonucleotide ligation assay, one or more of the oligonucleotides are labeled, in other synthesis reactions, either the primer or the nucleotides are typically labeled.

Commonly employed labels include, but are not limited to, biotin, fluorescent molecules, radioactive molecules, chromogenic substrates, chemi-luminescence, and the like. The methods for biotinylating nucleic acids are well known in the art, as are methods for introducing fluorescent molecules and radioactive molecules into oligonucleotides and nucleotides.

When biotin is employed, it is detected by avidin, streptavidin or the like, which is conjugated to a detectable marker, such as an enzyme (e.g., horseradish peroxidase) or radioactive label (e.g., ^{32}P, ^{35}S, ^{33}P). Enzyme conjugates are commercially available from, for example, Vector Laboratories (Burlingame, Calif.). Steptavidin binds with high affinity to biotin, unbound stretavidin is washed away, and the presence of horseradish peroxidase enzyme is then detected using a precipitating substrate in the presence of peroxide and appropriate buffers. The product may be detected using a microscope equipped with a visible light source and a CCD camera (Princeton Instruements, Princeton, N.J.). With such an instrument, an image of approximately 10,000 μM×10,000 μM can be scanned at one time.

Detection methods are well known for fluorescent, radioactive, chemiluminescent, chromogenic labels, as well as other commonly used labels. Briefly, fluorescent labels can be identified and quantitated most directly by their absorption and fluorescence emission wavelengths and intensity. A microscope/camera setup using a light source of the appropriate wave length is a convenient means for detecting fluorescent label. Radioactive labels may be visualized by standard autoradiography, phophor image analysis or CCD detector. Other detection systems are available and known in the art. For labels such as biotin, radioactive, or fluorescent, the number of different reactions that can be detected at a single time is limited. For example, the use of four fluorescent molecules, such as commonly employed in DNA sequence analysis, limits anaylsis to four samples at a time. Essentially, because of this limitation, each reaction must be individually assessed when using these detector methods.

A more advantageous method of detection allows pooling of the sample reactions on at least one array and simultaneous detection of the products. By using a tag having a different molecular weight or other physical attribute in each reaction, the entire set of reaction products can be harvested together and analyzed. (see U.S. application Ser. Nos. 08/786,835; 08/786,834; 08/787,521; 08/898,180; 08/898,564; 08/898,501 and International Publication Nos. 97/27331; 97/27325 and 97/27327, all incorporated herein by reference in their entireties). Briefly, a "tag" molecule is used as a label. As used herein, a "tag" refers to a chemical moiety which is used to uniquely identify a "molecule of interest", and more specifically refers to the tag variable component as well as whatever may be bonded most closely to it in any of the tag reactant, tag component and tag moiety.

A tag useful in the present invention possesses several attributes: (1) It is capable of being distinguished from all

other tags. This discrimination from other chemical moieties can be based on the chromatographic behavior of the tag (particularly after the cleavage reaction), its spectroscopic or potentiometric properties, or some combination thereof. Spectroscopic methods by which tags are usefully distinguished include mass spectroscopy (MS), infrared (IR), ultraviolet (UV), and fluorescence, where MS, IR and UV are preferred, and MS most preferred spectroscopic methods. Potentiometric amperometry is a preferred potentiometric method. (2) The tag is capable of being detected when present at 10^{-22} to 10^{-6} mole. (3) The tag possesses a chemical handle through which it can be attached to the MOI which the tag is intended to uniquely identify. The attachment may be made directly to the MOI, or indirectly through a "linker" group. (4) The tag is chemically stable toward all manipulations to which it is subjected, including attachment and cleavage from the MOI, and any manipulations of the MOI while the tag is attached to it. (5) The tag does not significantly interfere with the manipulations performed on the MOI while the tag is attached to it. For instance, if the tag is attached to an oligonucleotide, the tag must not significantly interfere with any hybridization or enzymatic reactions (e.g., amplification reactions) performed on the oligonucleotide.

A tag moiety that is intended to be detected by a certain spectroscopic or potentiometric method should possess properties which enhance the sensitivity and specificity of detection by that method. Typically, the tag moiety will have those properties because they have been designed into the tag variable component, which will typically constitute the major portion of the tag moiety. In the following discussion, the use of the word "tag" typically refers to the tag moiety (i.e., the cleavage product that contains the tag variable component), however can also be considered to refer to the tag variable component itself because that is the portion of the tag moiety which is typically responsible for providing the uniquely detectable properties. In compounds of the formula T—L—X, the "T" portion contains the tag variable component. Where the tag variable component has been designed to be characterized by, e.g., mass spectrometry, the "T" portion of T—L—X may be referred to as T^{ms}. Likewise, the cleavage product from T—L—X that contains T may be referred to as the T^{ms}-containing moiety. The following spectroscopic and potentiometric methods may be used to characterize T^{ms}-containing moieties.

Thus, within one aspect of the present invention, methods are provided for determining the identity of a nucleic acid molecule or fragment (or for detecting the presence of a selected nucleic acid molecule or fragment), comprising the steps of (a) generating tagged nucleic acid molecules from one or more selected target nucleic acid molecules, wherein a tag is correlative with a particular nucleic acid molecule and detectable by non-fluorescent spectrometry or potentiometry, (b) separating the tagged molecules by size (e.g., HPLC, electrophoresis) to remove labeled material not incorporated in the enzymatically generated product, (c) cleaving the tags from the tagged molecules, and (d) detecting the tags by non-fluorescent spectrometry or potentiometry, and therefrom determining the identity of the nucleic acid molecules. Examples of such technologies include for example mass spectrometry, infra-red spectrometry, potentiostatic amperometry or UV spectrometry.

IV. Uses

As noted above, the methods described herein may be used in a variety of ways. For example, amplification of template nucleic acids may be used for genotyping individuals, for mutation scanning, for determining expression profiles, and the like. Oligonucleotide ligation assays and single nucleotide extension assays may be used for mutation analysis, detection of a nucleic acid in a sample and the like. Each of these uses is briefly discussed below.

A. Genotyping

Within one preferred aspect of the present invention, methods are provided for genotyping a selected organism, comprising the steps of (a) generating tagged nucleic acid molecules from a selected target molecule, wherein a tag is correlative with a particular fragment and may be detected by non-fluorescent spectrometry or potentiometry, (b) separating the tagged molecules, (c) cleaving the tag from the tagged molecule, and (d) detecting the tag by non-fluorescent spectrometry or potentiometry, and therefrom determining the genotype of the organism. In other embodiments, the tag can be fluorescent, radioactive, etc.

Within another embodiment of the invention, methods are provided for determining the identity of a nucleic acid molecule, or for detecting a selecting nucleic acid molecule, in for example a biological sample, utilizing the technique of DNA fingerprinting. Briefly, such methods generally comprise the steps of generating a series of tagged nucleic acid fragments, followed by separation of the fragments by size. The size separation step can be accomplished, for example by gel electrophoresis (e.g., polyacrylamide gel electrophoresis) or preferably HPLC. The tags are then cleaved from the separated fragments, and then the tags are detected by the respective detection technology (e.g., mass spectrometry, infra-red spectrometry, potentiostatic amperometry or UV spectrometry).

Descriptions of many types of DNA sequence polymorphisms have provided the fundamental basis for the understanding of the structure of the human genome (Botstein et al., *Am. J. Human Genetics* 32:314, 1980; Donis-Keller, *Cell* 51:319, 1987; Weissenbach et al., *Nature* 359:794). The construction of extensive framework linkage maps has been facilitated by the use of these DNA polymorphisms and has provided a practical means for localization of disease genes by linkage. In addition to single base mutations, length variations of tandem repeats are also common in the genome, with at least tens of thousands of interspersed polymorphic sites (termed loci). There are two major groups of tandem repeat polymorphisms: minisatellites/variable number of tandem repeats (VNTRs), with typical repeat lengths of tens of base pairs and with tens to thousands of total repeat units, and microsatellites, with repeat lengths of up to 6 bp and with maximum total lengths of about 70 bp. Microsatellite dinucleotide repeats are proving to be very powerful tools in the identification of human genes, are highly polymorphic (Weber, 1990, Genomic Analysis, 1:159–181, Cold Spring Laboratory Press, Cold Spring Harbor, N.Y.; Weber and Wong, *Hum. Mol. Genetics*, 2:, 1123, 1993) and may possess up to 24 alleles. Chromosome specific markers which permit a high level of multiplexing have been reported for performing whole genome scans for linkage analysis (Davies et al., *Nature*, 371: 130, 1994).

Repeats can be amplified using primers complementary to the unique regions surrounding the dinucleotide repeat. Following amplification, several amplified loci can be combined (multiplexed) prior to capture on an array.

Genotyping or DNA fingerprinting involves the display of a set of DNA fragments from a specific sample. A variety of DNA fingerprinting techniques are presently available (Jeffreys et al., *Nature*, 314: 67–73, 1985: Zabeau and Vos, European Patent Application 92402629.7.; Vos et al. *Nucl.*

Acids Res. 23: 4407–4414, 1996; Bates et al., in *The Impact of Plant Molecular Genetics*, Chapter 14, pp. 239–255, ed. B.W.S. Sobral, Birkhauser Publishing). DNA fingerprinting involves the display of a set of DNA fragments from a specific DNA sample. A variety of DNA fingerprinting techniques are presently available (Jeffries et al., *Nature* 314:67, 1985; Welsh and McClelland, *Nuc. Acids. Res.* 19:861, 1991), most of which use amplification (e.g., PCR) to generate fragments. The DNA fingerprinting process produces "fingerprint" patterns of different fragment lengths that are characteristic and reproducible for an individual organism. These fingerprints can be use to distinguish even very closely related organisms, including near-isogenic lines. The differences in fragment lengths or sequence can be traced to base changes in the restriction site or the primer extension site, or to insertions or deletions within a DNA fragment.

The choice of which fingerprinting technique to use is dependent on the application, (e.g., DNA typing, DNA marker mapping) and the organisms under investigation, (e.g., prokaryotes, plants, animals, humans). A number of fingerprinting methods which meet these requirements have been developed, including random amplified polymorphic DNA (RAPD), DNA amplification fingerprinting (DAF), and arbitrarily primed PCR (AP-PCR). These methods are all based on the amplification of random genomic DNA fragments by arbitrarily selected PCR primers allowing generation of DNA fragment patterns from any DNA without prior sequence knowledge. The patterns generated depend on the sequence of the amplification primers and the nature of the template DNA. Low annealing temperatures are used to allow the primers to anneal to multiple loci on the DNA, which are amplified when primer binding sites are sufficiently close together. In principle, a single primer is sufficient for generating band patterns.

An additional technique for DNA fingerprinting has been described, named AFLP (Vos et al., *Nuc. Acids Res.* 23:4407, 1995). The AFLP technique is based on the detection of genomic restriction fragments by amplification, and can be used for DNAs of any origin or complexity. Briefly, the technique is based on selective amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: 1) restriction of the DNA fragments and subsequent ligation of oligonucleotide adaptors, 2) selective amplification of sets of restriction fragments, 3) analysis of the amplified fragments. Amplification of the restriction fragments is achieved by using the adaptor and restriction site sequence as target sites for primer annealing. The selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. This method therefore yields sets of restriction fragments which may be visualized by a variety of methods (e.g., PAGE, HPLC, or other types of spectrometry) without prior knowledge of the nucleotide sequence. The method also allows the co-amplification of large numbers of restriction fragments. The number of fragments however is dependent on the resolution of the detection system. Typically, 50–100 restriction fragments are amplified and detected.

An amplification approach to identify restriction fragment length polymorphism (RFLP) combines separation techniques with detection of tags associated with specific PCR primers. In general, one primer will possess one specific tag. The tag will therefore represent one set of primers and therefore a pre-determined DNA fragment length. Polymorphisms are detected as variations in the lengths of the labeled fragments in a gel or eluting from a gel. HPLC or polyacrylamide gel electrophoresis will usually afford the resolution necessary to distinguish minisatellite/VNTR alleles differing by a single repeat unit. Analysis of microsatellite polymorphisms involves amplification by the polymerase chain reaction (PCR) of a small fragment of DNA containing a block of repeats followed by electrophoresis of the amplified DNA on denaturing polyacrylamide gel or followed by separation of DNA fragments by HPLC. The amplified DNA may be labeled using primers that have labels attached. The primers are incorporated into the newly synthesized strands by chain extension. The primers are complementary to unique sequences that flank the blocks of repeats.

Tags can be used to great effect in genotyping with microsatellites. Briefly, the PCR primers are constructed to carry tags and used in a carefully chosen PC reaction to amplify di-, tri-, or tetra-nucleotide repeats. The amplification products are then separated according to size by methods such as HPLC or PAGE. The DNA fragments are then collected in a temporal fashion, the tags cleaved from their respective DNA fragments and length deduced from comparison to internal standards in the size separation step. Allele identification is made from reference to size of the amplified products.

By using cleavable tags in genotyping, it is possible to combine multiple samples on a single separation step. There are two general ways in which this can performed. The first general method for high through-put screening is the detection of a single polymorphism in a large group of individuals. In this scenario, a single or nested set of PCR primers is used and each amplification is done with one DNA sample type per reaction. The number of samples that can be combined in the separation step is proportional to the number of cleavable tags that can be generated per detection technology (i.e., 400–600 for mass spectrometer tags). It is therefore possible to identify several polymorphisms in a large group of individuals simultaneously. The second approach is to use multiple sets of primers which can identify numerous polymorphisms on a single DNA sample (genotyping an individual for example). In this approach, primers are combined in a single amplification reaction which generate amplified products of different sequence. Each primer pair or nested set is encoded by a specific cleavable tag resulting in each amplified fragment encoded with a specific tag. The reaction is run on a single separation step. The number of samples that can be combined in the separation step is proportional to the number of cleavable tags that can be generated per detection technology (i.e., 400–600 for mass spectrometer tags).

B. Mutation detection

The detection of diseases is increasingly important in prevention and treatments. While multifactorial diseases are difficult to devise genetic tests for, more than 200 known human disorders are caused by a defect in a single gene, often a change of a single amino acid residue (Olsen, *Biotechnology: An industry comes of age*, National Academic Press, 1986). Many of these mutations result in an altered amino acid that causes a disease state.

Sensitive mutation detection techniques offer extraordinary possibilities for mutation screening. For example, analyses may be performed even before the implantation of a fertilized egg (Holding and Monk, *Lancet* 3:532, 1989). Increasingly efficient genetic tests may also enable screening for oncogenic mutations in cells exfoliated from the respiratory tract or the bladder in connection with health checkups (Sidransky et al., *Science* 252:706, 1991). Also, when an unknown gene causes a genetic disease, methods to monitor

DNA sequence variants are useful to study the inheritance of disease through genetic linkage analysis. However, detecting and diagnosing mutations in individual genes poses technological and economic challenges. Several different approaches have been pursued, but none are both efficient and inexpensive enough for truly wide-scale application.

Mutations involving a single nucleotide can be identified in a sample by physical, chemical, or enzymatic means. Generally, methods for mutation detection may be divided into scanning techniques, which are suitable to identify previously unknown mutations, and techniques designed to detect, distinguish, or quantitate known sequence variants. Several scanning techniques for mutation detection have been developed based on the observation that heteroduplexes of mismatched complementary DNA strands, derived from wild type and mutant sequences, exhibit an abnormal migratory behavior.

One strategy for detecting a mutation in a DNA strand is by substituting (during synthesis) one of the normal nucleotides with a modified or labeled nucleotide or by altering the molecular weight or other physical parameter of the product. A strand with an increased or decreased number of this modified nucleotide relative to the wild-type sequence exhibits altered mobility (Naylor et al., *Lancet* 337:635, 1991). Heteroduplex DNA molecules generated by amplification, containing internal mismatches, can also be separated from correctly matched molecules by mobility (Orita, *Genomics* 5:874, 1989; Keen, *Trends Genet.* 7:5, 1991), indicating the presence of a mutation in a limited segment of DNA.

Mutations may be also be identified via their destabilizing effects on the hybridization of short oligonucleotide probes to a target sequence (see Wetmur, *Crit. Rev. Biochem. Mol. Biol.*, 26:227, 1991). Generally, this technique, allele-specific oligonucleotide hybridization, involves amplification of target sequences and subsequent hybridization with short oligonucleotide probes. An amplified product can thus be scanned for many possible sequence variants by determining its hybridization pattern to an array of immobilized oligonucleotide probes. Another method exploits the property that an oligonucleotide primer that is mismatched to a target sequence at the 3' penultimate position exhibits a reduced capacity to serve as a primer in PCR. Additional mismatches may be incorporated into the primer at the third position from the 3' end. This results in two mismatched positions in the three 3' nucleotides of the primer hybridized with one allelic variant, and one mismatch in the third position in from the 3' end when hybridized to the other allelic variant (Newton et al., *Nucl. Acids Res.* 17:2503, 1989). Amplification conditions are chosen that significantly favor amplification of a 1 bp mismatch.

C. Expression profiles/differential display

Mammals, such as human beings, have about 100,000 different genes in their genome, of which only a small fraction, perhaps 15%, are expressed in any individual cell. The process of normal cellular growth and differentiation, as well as the pathological changes that arise in diseases like cancer, are all driven by changes in gene expression. Differential display techniques permit the identification of genes specific for individual cell types.

As disclosed herein, a high throughput method for measuring the expression of numerous genes (1–2000) is provided. Within one aspect of the invention methods are provided for analyzing the pattern of gene expression from a selected biological sample, comprising the steps of (a) amplifying cDNA from a biological sample using one or more tagged primers, wherein the tag is correlative with a particular nucleic acid probe and detectable by non-fluorescent spectrometry, or potentiometry, (b) separating amplified fragments, (c) cleaving the tag from the tagged fragment, and (d) detecting the tag by non-fluorescent spectrometry, or potentiometry, and therefrom determining the pattern of gene expression of the biological sample.

Briefly, in differential display, the 3' terminal portions of mRNAs are amplified and identified on the basis of size. Using a primer designed to bind to the 5' boundary of a poly(A) tail for reverse transcription, followed by amplification of the cDNA using upstream arbitrary sequence primers, mRNA sub-populations are obtained. Size separation methods (PAGE, HPLC, etc.) allows direct side by side comparison of lengths or amounts of the mRNAs between two biological samples of interest. The differential display method has the potential to visualize all the expressed genes (about 10,000 to 15,000 mRNA species) in a mammalian cell by using multiple primer combinations.

Tag-based differential display on solid substrates allows characterization of differentially expressed genes. It is based on the principle that most mRNAs expressed in two or more cell types or samples of interest can be directly compared on gels by amplifying partial cDNA sequences from subsets of mRNA with reverse transcription and PCR. Briefly, three one-base anchored oligo-dT primers are used in combination with a series of arbitrary 13 base oligonucleotides to reverse transcribe and amplify the mRNAs from a cell or sample of interest. For monitoring the expression of 15,000 genes, it is preferred that at least nine different arbitrary primers are used. For a complete differential display analysis of two cell populations or two samples of interest, at least 400 amplification reactions are required. With tag-based differential display analysis of two cell types, at least 1500 amplification reactions are easily and quickly performed.

D. Single nucleotide extension assay

The primer extension technique may be used for the detection of single nucleotide in a nucleic acid template (Sokolov, *Nucleic Acids Res.*, 18:3671, 1989). As originally described, 30 base oligonucleotides and 20 base oligonucleotides complementary to the known sequence of the cystic fibrosis gene were extended in the presence of a single labeled nucleotide. The method had the ability to correctly identify a single nucleotide change within the gene. The technique is generally applicable to detection of any single base mutation (Kuppuswamy et al., *Proc. Natl. Acad. Sci. USA*, 88:1143–1147, 1991).

Briefly, this method is based on a primer that hybridizes to a sequence in a target molecule adjacent to a known single nucleotide polymorphism. Within the context of the present invention, the target molecule is preferably covalently attached to the solid substrate. The primed DNA is then subjected to conditions in which a DNA polymerase adds a labeled dNTP, or ddNTP, if the next base in the template is complementary to the labeled nucleotide in the reaction mixture. Free labeled dNTP or ddNTP is washed away, and the extended products are detected.

In a modification of the technique, cDNA is a template for amplification of a sequence of interest containing a single-base difference between two alleles. The amplification products are then printed on the array. Each amplified product is then analyzed for the presence, absence, or relative amounts of each allele by annealing a primer that is 1 base 5' to the polymorphism and extending by one labeled base (generally a dideoxynucleotide). Only when the correct base is available in the reaction will incorporation occur at the 3'-end of the primer. Extension products are then analyzed as above.

In the present invention, each (di)deoxynucleotide is labeled with a unique tag. Of the four reaction mixtures, only

one will add a dideoxy-terminator on to the primer sequence. If the mutation is present, it will be detected through the unique tag on the dideoxynucleotide and its identity established. Multiple mutations can be ascertained simultaneously by tagging the DNA primer with a unique tag as well. Thus, the DNA fragments are reacted in four separate reactions each including a different tagged (di) deoxyterminator, wherein the tag is correlative with a particular dideoxynucleotide and detectable by non-fluorescent spectrometry, or potentiometry. The DNA fragments are separated according to size by, for example, gel electrophoresis (e.g., polyacrylamide gel electrophoresis) or preferably HPLC or detected in situ. The tags are cleaved from the fragments and detected by the respective detection technology (e.g., mass spectrometry, infrared spectrometry, potentiostatic amperometry or UV/visible spectrophotometry). The tags detected can be correlated to the particular DNA fragment under investigation as well as the identity of the mutant nucleotide.

E. Oligonucleotide ligation assay

The oligonucleotide ligation assay (OLA) as originally described by Landegren et al. (Landegen et al., *Science* 241:487, 1988) is used for the identification of known sequences in very large and complex genomes. The principle of OLA is based on the ability of ligase to covalently join two diagnostic oligonucleotides as they hybridize adjacent to one another on a given DNA target. If the sequences at the probe junctions are not perfectly based-paired, the probes will not be joined by the ligase. The ability of a thermostable ligase to discriminate potential single base-pair differences when positioned at the 3' end of the "upstream" probe provides the opportunity for single base-pair resolution (Barony, *Proc. Natl. Acad. Sci. USA*, 88:189, 1991). When tags are used, they are attached to the probe, which is ligated to the amplified product. After completion of OLA, unligated oligo nucleotides are removed by incubation at a temperature that melts the unligated oligonucleotides but not the ligated oligonucleotides. Althematively, fragments are separated on the basis of size. The tags are cleaved and detected by mass spectrometry.

In another embodiment, oligonucleotide-ligation assay employs two adjacent oligonucleotides: a "reporter" probe (tagged at the 5' end) and a 5'-phosphorylated/3' tagged "anchor" probe. The two oligonucleotides, which have incorporated different tags, are annealed to target DNA and, if there is perfect complementarity, the two probes are ligated by T4 DNA ligase. In one embodiment, the 3' tag is biotin and capture of the biotinylated anchor probe on immobilized streptavidin and analysis for the covalently linked reporter probe test for the presence or absence of the target sequences.

Within one embodiment of the invention methods are provided for determining the identity of a nucleic acid molecule, or for detecting a selecting nucleic acid molecule, in, for example a biological sample, utilizing the technique of oligonucleotide ligation assay. Briefly, such methods generally comprise the steps of performing amplification on the target DNA followed by hybridization with the 5' tagged reporter DNA probe and a 5' phosphorylated/non-biotinylated probe. The sample is incubated with T4 DNA ligase. The DNA strands with ligated probes can be separated from the DNA with non-ligated probes by, for example, preferably by LC or HPLC. The tags are cleaved from the separated fragments, and then the tags are detected by the respective detection technology (e.g., mass spectrometry, infrared spectrophotometry, potentiostatic amperometry or UV/visible spectrophotometry.

In the present invention, multiple samples and multiple mutations may be analyzed concurrently. Briefly, the method consists of amplifying the gene fragment containing the mutation of interest. The amplified product is then hybridized with a common and two allele-specific oligonucleotide probes (one containing the mutation while the other does not) such that the 3' ends of the allele-specific probes are immediately adjacent to the 5' end of the common probe. This sets up a competitive hybridization-ligation process between the two allelic probes and the common probe at each locus. The common probe is labeled with one of four fluorophores and the allele-specific probes are each labeled with one or more tags that provide sizing differences. The samples are then separated based upon the length of the modifying tails and detected by the fluorescent tag on the common probe. Through the use in sizing differences on the allele-specific probes and four fluorophores available for the common probe, many samples can be analyzed.

Within one embodiment of the invention methods are provided for determining the identity of a nucleic acid molecule, or for detecting a selecting nucleic acid molecule, in, for example a biological sample, utilizing the technique of oligonucleotide ligation assay for concurrent multiple sample detection. Briefly, such methods generally comprise the steps amplifying target DNA followed by hybridization with the common probe (untagged) and two allele-specific probes tagged according to the specifications of the invention. The sample is incubated with DNA ligase and fragments separated by, for example, preferably by LC or HPLC. The tags are cleaved from the separated fragments, and then the tags are detected by the respective detection technology (e.g., mass spectrometry, infrared spectrophotometry, potentiostatic amperometry or UV/visible spectrophotometry.

F. Other assays

The methods described herein may also be used to genotype or identification of viruses or microbes. For example, F+RNA coliphages may be useful candidates as indicators for enteric virus contamination. Genotyping by nucleic acid amplification and hybridization methods are reliable, rapid, simple, and inexpensive alternatives to serotyping (Kafatos et. al., *Nucleic Acids Res.* 7:1541, 1979). Amplification techniques and nucleic aid hybridization techniques have been successfully used to classify a variety of microorganisms including *E. coli* (Feng, *Mol. Cell Probes* 7:151, 1993), rotavirus (Sethabutr et. al., *J. Med Virol.* 37:192, 1992), hepatitis C virus (Stuyver et. al., *J. Gen Virol.* 74:1093, 1993), and herpes simplex virus (Matsumoto et. al., *J. Virol. Methods* 40:119, 1992).

Genetic alterations have been described in a variety of experimental mammalian and human neoplasms and represent the morphological basis for the sequence of morphological alterations observed in carcinogenesis (Vogelstein et al., *NEJM* 319:525, 1988). In recent years with the advent of molecular biology techniques, allelic losses on certain chromosomes or mutation of tumor suppressor genes as well as mutations in several oncogenes (e.g., c-myc, c-jun, and the ras family) have been observed. For example, a correlation between specific types of point mutations in the K-ras oncogene and the stage at diagnosis in colorectal carcinoma has been identified (Finkelstein et al., *Arch Surg.* 128:526, 1993). Thus, mutational analysis could provide important information of tumor aggressiveness, including the pattern and spread of metastasis. Indeed, the prognostic value of TP53 and K-ras-2 mutational analysis in stage III carcinoma of the colon has been demonstrated (Pricolo et al., *Am. J. Surg.* 171:41, 1996). It is therefore apparent that genotyping of tumors and pre-cancerous cells, as well as specific muta-

tion detection will become increasingly important in the treatment of cancers in humans.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

Example 1

Preparation of Arraying Tip from a Commercial Spring Probe.

This example describes the manufacture and modification of a spring probe tip for use in depositing samples in an array.

XP54P spring probes are purchased from Osby-Barton (a division of Everett Charles (Pomona, Calif.)). The probes are placed "tip-down" on an extra fine diamond shrpening stone and moved across the stone about 0.5 cm with gentle pressure. Approximately 0.005 inches (0.001 to 0.01 inches) of metal is removed from the end of the tip as observed by microscopy. The tip end is polished by rubbing the tip across a leather strip and then washed with water. Tips are stored dry or stored in 50% glycerol at −20° C. For use in preparation of arrays, the tips are mounted in a head in an array fashion. The head is mounted on an robotic arm, which possesses controllable motion in the z-axis.

Example 2

Preparation of Arrays of Microspheres of Glass Slides.

Deposition of easily detectable microspheres on glass slides demonstrates reproducibility of array formation. In this procedure, a solution consisting of 56% glycerol, 0.01 M Tris pH 7.2, 5 mM EDTA, 0.01% sarkosyl, and 1% v/v Fluoresbrite Plain 0.5 μM microspheres (2.5% solids-latex), (Polysciences, Warrington, Pa.) is prepared. An arraying pin is submerged 5 mm into this solution for 5 sec. The microspheres are then repeatedly arrayed onto a glass slide. Photomicrographs of the slide are taken under fluorescence light using a filter for fluorescence. FIG. 1 demonstrates that the amount of deposited solution in each area of the array is very consistent. Moreover, at least 100 deposits can be made per pickup that are virtually identical.

Example 3

Preparation of as Array Using a Modified Hydrophilic Spring Probe

Sample pick-up, transfer and micro-droplet deposition is greatly enhanced when using a liquid transfer device that has a hydrophilic surface, especially when that device is a modified spring probe. Spring probes are rendered hydrophilic through the use of chemical agents acting to modify the surface of the probe or through coating the probe with a hydrophilic substance. In a preferred method, the tip of the spring probe is soaked in a 25–200 mM solution of 1,4-dithiothreitol, 0.1 M sodium borate for 15 min to 2 hrs. Dithiothreitol reacts with gold surfaces through a thiol-gold coordination, which essentially hydroxylates the surface, making it hydrophilic.

An arraying solution is made consisting of 56% glycerol and 44% water colored with blue food color. The arraying tip is submerged 5 mm into the arraying solution for 2 sec. The glycerol bearing tip is then robotically controlled to print 72 microspots in a 12×6 grid onto a silicon wafer. The spots produced were approximately 100–150 microns in diameter with 200 micron center to center spacing between spots. FIG. 2 shows a CCD camera image of the grid produced. The standard deviation of spot diameter is approximately 15%.

Example 4

Colorimetric Detection of Arrayed Oligonucleotides.

Template oligonucleotide (75 μl of 0.5 μg/μl) (5'- hexylamine GTCATACTCCT-GCTTGCTGATCCACATCTG-3') (SEQ ID NO: 1) is reacted with 5 μl of a 20 mg/ml cyanuric chloride in 20 μl of 1 M sodium borate for 30 min at room temperature. From this reaction, an arraying solution is made, which consists of 56% glycerol, 56 ng/ul oligonucleotide, 0.06 mM sodium borate and 0.3 mg/ml cyanuric chloride. The arraying tip is submerged 5 mm into the arraying solution for 2 sec. The solution bearing tip is then robotically controlled to print 72 microspots in a 12×6 grid onto a polyethyleneimine (PEI) coated silicon wafer. The spots produced are approximately 100–150 microns in diameter with 200 micron center to center spacing between spots. Following arraying, the unreacted PEI sites on the wafer are blocked with 100 mg/ml succinic anhydride in 100% n-methyl pyrrolinidone for 15 minutes followed by 3 washes in water. The unreacted cyanuric chloride sites are blocked with 0.1 M glycine in 0.01 M Tris for 15 minutes with four washes in Tens buffer (0.1 M NaCl, 0.1% SDS, 0.01 M Tris, 5 mM EDTA). The template oligomer is then hybridized to its biotinylated complement (5'-Biotin-TGTGGATCAGCAAGCAGGAGTATG-3') (SEQ ID NO:2) for 20 min at 37° C. followed by a wash in 6× Tens and 2× OHS (0.06 M Tris, 2 mM EDTA, 5× Denhardt's solution, 6× SSC [3 M NaCl, 0.3 M sodium citrate, pH 7.0], 3.68 mM N-lauroylsarcosine, 0.005% NP-40). The wafer is then soaked in 0.5 μg/ml alkaline phosphatase conjugated streptavidin for 15 min followed by a wash in 2× Tens, 4× TWS (0.1 M NaCl, 0.1% Tween 20, 0.05 M Tris). The microspots are then developed using Vector Blue (Vector Laboratories, Burlingame, Calif.) (following kit protocol) and imaged with a CCD camera and microscope. FIG. 3 displays the image generated. The resulting microspots have approximately a 15% variation in diameter and intensity values varying approximately 10% as determined by NIH Image (National Institute of Health, Bethesda, Md.).

Example 5

Multiple Oligos Within a Single Array Element

Two template oligos (#1, 5'-hexylamine-TGTGGATCAGCAAGCAGG AGTATG-3'(SEQ ID NO:2), #2 5'-hexylamine-ACTACTGATCAGGCGCGCCTTTTTTTTTTTTTTT TTTF3') (SEQ ID NO:3) at 0.5 μg/μl are reacted seperately with 5 μl of 20 mg/ml cyanuric chloride and 20 μl of 1M sodium borate in a total reaction volume of 100 μl for 30 minutes at room temperature. From these two reactions, arraying solutions are made of 56% glyceroland diluted combinations of the two reacted oligos (see Table below). Eight arraying tips are submerged 5 millimeters into each of the eight arraying solutions for 2 seconds. The solution bearing tips are robotically controlled to print two sets of eight 12×6 grids each containing 72 microspots onto a polyethyleneimine (PEI) coated silicon wafer. Each grid represents a single arraying solution. The spots produced are approximately 100–150 microns in diameter with 200 micron center to center spacing between spots.

Following arraying, the unreacted PEI sites on the wafer are blocked with 100 mg/ml succinic anhydride in 100% n-methyl pyrrolinidone for 15 minutes with a 3× water wash. The unreacted cyanuric chloride sites are blocked with 0.1 M glycine in 0.01 M Tris for 15 minutes with a 4× Tens (0.1 M NaCl, 0.1% SDS, 0.01 M Tris, 5 mM EDTA) wash. Two hybridizations are then carried out. In the first hybridization, one set of the eight arrayed oligo combinations is hybridized to the oligonucleotide, 5'-Biotin-

TGTGGATCAGCAAGCAGGAGTATG-3'(SEQ ID NO: 2), which is complementry to oligo #1. In the second hybridization, the other set of the eight arrayed oligo combinations is hybridized to the oligonucleotide (5'-BIOTIN-AAAAAA AAAAAAAAAAAAAAGGCGCGCCTGATCAGTAGT) (SEQ ID NO:4), which is complementry to oligo #2. The hybridizations are conducted simultaneously under Hybriwell Sealing Covers (Research Products International Corporation, Mount Prospect, Ill.) for 20 minutes at 37° C. followed by a 6× Tens, 2× OHS (0.06 M Tris, 2 mM EDTA, 5× Denhardt's solution, 6× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0), 3.68 mM N-lauroylsarcosine, 0.005% NP-40) wash. Following hydridization, the wafer is soaked in 0.5 μg/ml horseradish peroxidase streptavidin for 15 minutes followed by a 2× Tens, 4× TWS (0.1 M NaCl, 0.1% Tween 20, 0.05 M Tris) wash. The microspots are then developed using 0.4 mg/ml 4-methoxy 1-napthol (0.02% hydrogen peroxide, 12% methanol, PBS) with a final 3× water wash.

The set of mixed oligos that hybridize to the complement of oligo #1 show the greatest color intensity for the grid containing the highest concentration of oligo #1 and the least color intensity with the grid containing the lowest concentration of oligo #1. Whereas, the set of mixed oligos hybridized to the complement of oligo #2, showed the greatest color intensity for the grid containing the highest concentration of oligo #2 and the least color intensity with the grid containing the lowest concentration of oligo #2 (see FIG. 4).

Arraying Solution	Concentration of oligo #1 in arraying solution (ng/μl)	Concentration of oligo #2 in arraying solution (ng/μl)
1	56	0.44
2	28	0.88
3	14	1.8
4	7	3.5
5	3.5	7
6	1.8	14
7	0.88	28
8	0.44	56

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

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What is claimed is:

1. A method of amplifying nucleic acid molecules from a template in a chamber, comprising:

(a) mixing single-stranded nucleic acid templates on a solid substrate with a solution comprising an oligonucleotide primer that hybridizes to the templates and a DNA polymerase, wherein the mixing occurs in discrete areas on the substrate, and wherein the solution remains in the discrete areas;

(b) synthesizing a complementary strand to the template to form a duplex;

(c) denaturing the duplex; and

(d) synthesizing complementary strands to the template, therefrom amplifying nucleic acid molecules;

wherein the nucleic acid solution is in contact with the atmosphere in the chamber, and dew point is maintained during said mixing, synthesizing, and denaturing, thereby preventing evaporation of the solution.

2. A method of amplifying nucleic acid molecules from a template in a chamber, comprising:

(a) mixing single-stranded nucleic acid templates on a solid substrate with a solution comprising a first oligonucleotide primer that hybridizes to the templates, a second oligonucleotide primer that hybridizes to a complementary strand of the template, and a DNA polymerase, wherein the mixing occurs in discrete areas on the substrate, and wherein the solution remains in the discrete areas;

(b) synthesizing a complementary strand to the template to form a duplex;

(c) denaturing the duplex; and

(d) synthesizing complementary strands to the template and the complementary strand of the template, therefrom amplifying nucleic acid molecules;

wherein the solution is in contact with the atmosphere in the chamber, and dew point is maintained during said mixing, synthesizing, and denaturing, thereby preventing evaporation of the solution.

3. The method of either of claims 1 or 2, wherein steps (c) and (d) are performed multiple times.

4. The method of claim 3, wherein steps (c) and (d) are performed from about 10 to about 25 times.

5. The method of either of claims 1 or 2, wherein the solution contains a compound that confers viscosity.

6. The method of claim 5, wherein the compound is glycerol or a sugar.

7. The method of claim 6, wherein the compound is glycerol.

8. The method of claim 6, wherein the compound is a sugar.

9. The method of either of claims 1 or 2, wherein the DNA polymerase is a thermostable polymerase.

10. The method of either of claims 1 or 2, wherein synthesis and denaturation are performed at different temperatures.

11. The method of either of claims 1 or 2, further comprising detecting the duplexes.

12. The method of claim 11, wherein the oligonucleotide primers are labeled.

13. The method of claim 12, wherein the label is a fluorescent molecule.

14. The method of claim 12, wherein the label is a tag that is detectable by non-fluorescent spectrometry or potentiometry.

15. The method of claim 14, wherein the detection of the tag is by mass spectrometry, infrared spectrometry, ultraviolet spectrometry, or poteniostatic amperometry.

16. The method of claim 14, wherein the sequence and the tag of the first or second or both oligonucleotide primers is different for each template.

17. The method of claim 16, wherein the amplified nucleic acids are pooled prior to detection.

18. The method of either of claims 1 or 2, wherein the array is on a solid substrate comprising a silicon wafer or borosilicate slide.

19. The method of claim 18, wherein the templates are covalently attached to the solid substrate.

20. The method of claim 19, wherein the attachment is through a polyethylene imine linkage.

21. The method of claim 2, wherein the oligonucleotide primer pairs each have a different sequence.

22. The method of either of claims 1 or 2, wherein the template is uniformly applied to the entire array prior to mixing.

23. The method of either of claims 1 or 2, wherein the template is applied individually to each discrete area on the substrate.

24. The method of claim 23, wherein the applying is performed using spring probes.

25. The method of either of claims 1 or 2, wherein an apparatus is used to control the dew point.

26. A method of synthesizing a nucleic acid molecule from a template, comprising:

(a) mixing single-stranded nucleic acid templates on a solid substrate with a solution comprising an oligonucleotide primer that hybridizes to the templates and a DNA polymerase, wherein the mixing occurs in a discrete area of an array, and wherein the solution remains in discrete areas; and

(b) synthesizing a complementary strand to the template to form a duplex,

wherein mixing and synthesis are performed at dew point,

wherein dew point is achieved by an apparatus, comprising: a container capable of being pressurized; a heating device; a means for generating pressure; and a means for generating saturated steam;

wherein the heating device, pressure generating means, and steam genearting means are controllable.

27. A method of detecting a single base alteration in a nucleic acid molecule, comprising:

(a) mixing single-stranded nucleic acid molecules on a solid substrate with a solution comprising a first and a

second oligonucleotides that hybridize to the nucleic acid molecules and a DNA ligase, wherein the mixing occurs in a discrete area of an array, and wherein the solution remains in the discrete areas; and

(b) detecting a ligation product;

wherein the first and second oligonucleotides will not ligate when there is a single base alteration at the junction base on the nucleic acid molecule, pl mixing is performed at dew point,

wherein dew point is achieved by an apparatus, comprising: a container capable of being pressurized; a heating device; a means for generating pressure; and a means for generating saturated steam;

wherein the heating device, pressure generating means, and steam generating means are controllable.

28. A method of performing single nucleotide extension assay, comprising:

(a) mixing oligonucleotides on a solid substrate with a solution comprising single-stranded nucleic acid molecules that hybridize to the oligonucleotides, a single nucleotide, and a DNA polymerase, wherein the mixing occurs in discrete areas of the substrate, and wherein the solution remains in discrete areas; and

(b) detecting an extension product of the oligonucleotide;

wherein the oligonucleotide will be extended only when the single nucleotide is complementary to the nucleotide adjacent to the hybridized oligonucleotide,

wherein mixing is performed at dew point,

wherein dew point is achieved by an apparatus, comprising: a container capable of being pressurized; a heating device; a means for generating pressure; and a means for generating saturated steam;

wherein the heating device, pressure generating means, and steam generating means are controllable.

29. The method of claim **26** wherein step (b) is performed multiple times.

30. The method of claim **26** wherein the solution contains a compound that increases the viscosity of the solution.

31. The method of claim **30** wherein the compound is glycerol or a sugar.

32. The method of claim **30** wherein the array is located on a substantially flat surface of a substrate.

33. The method of claim **32** wherein the substrate is glass.

34. The method of claim **27** wherein the solution contains a compound that increases the viscosity of the solution.

35. The method of claim **34** wherein the compound is glycerol or a sugar.

36. The method of claim **27** wherein the nucleic acid molecules form an array on the substrate, and the array is located on a substantially flat surface of the substrate.

37. The method of claim **36** wherein the substrate is glass.

38. The method of claim **28** wherein the solution contains a compound that increases the viscosity of the solution.

39. The method of claim **38** wherein the compound is glycerol or a sugar.

40. The method of claim **28** wherein the oligonucleotides form an array on the substrate, and the array is located on a substantially flat surface of the substrate.

41. The method of claim **40** wherein the substrate is glass.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,248,521 B1 Page 1 of 1
DATED : June 19, 2001
INVENTOR(S) : Jeffrey Van Ness et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [56], **References Cited**, page 2 of the Issued Patent, reference "cmgm.stanford.edu/pbrown/mguide, Sep. 12, 2000 and emgm.stanford.edu/pbrown/mguide/tips. Sept. 12, 2000." should read -- http://cmgm.stanford.edu/pbrown/mguide, 09/12/00 and http://cmgm.stanford.edu/pbrown/mguide/tips.html, 09/12/00 --.

Column 25, claim 27,
Line 9, "molecule, p l mixing" should read -- molecule, wherein mixing --.

Signed and Sealed this

Fifth Day of March, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

US006150102A

United States Patent [19]

Mills, Jr. et al.

[11] Patent Number: 6,150,102

[45] Date of Patent: Nov. 21, 2000

[54] METHOD OF GENERATING NUCLEIC ACID OLIGOMERS OF KNOWN COMPOSITION

[75] Inventors: Allen P. Mills, Jr., Chatham; Bernard Yurke, Plainfield, both of N.J.

[73] Assignee: Lucent Technologies Inc., Murray Hill, N.J.

[21] Appl. No.: 09/078,761

[22] Filed: May 15, 1998

Related U.S. Application Data

[63] Continuation-in-part of application No. 09/018,248, Feb. 3, 1998.

[51] Int. Cl.[7] C12Q 1/68; C12P 19/34; G01N 15/06; G01N 27/00; B32B 19/02

[52] U.S. Cl. 435/6; 435/91.1; 435/91.2; 422/68.1; 422/50; 422/63; 422/82.01; 422/82.02; 422/82.12; 422/131; 422/138

[58] Field of Search 435/6, 91.1, 91.2; 422/68.1, 50, 63, 82.01, 82.02, 82.12, 131, 138

[56] References Cited

U.S. PATENT DOCUMENTS

5,412,087	5/1995	McGall et al.	536/24.3
5,445,934	8/1995	Fodor et al.	435/6
5,503,980	4/1996	Cantor	435/6
5,561,071	10/1996	Hollenberg et al.	437/1
5,605,662	2/1997	Heller et al.	422/68.1

OTHER PUBLICATIONS

Corn, DNA Computing Overview, last modified Mar. 13, 1998, <http://www.corninfo.wisc.edu/writings/DNAoverview.html>).

Pall Ultrafine Filtration Corporation, "Protocols for DNA and RNA Transfer, DNA Electrotransfer, and Protein Transfer to Biodyne, A Nylon Membranes," 1983, East Hills, NY, pp. 3–5, and 14–15.

A. Marshall et al., "DNA chips: An array of possibilities," Nature Biotechnology, vol. 16, Jan. 1998, pp. 27–31.

Graham Ramsay, "DNA chips: State-of-the art", Nature Biotechnology, vol. 16., Jan. 1998, pp. 40–44.

Leonard M. Adleman, "Molecular Computation of Solutions to Combinatorial Problems", Science, vol. 226, Nov. 11, 1994, pp. 1021–1024.

John S. Oliver, "Matrix Multiplication with DNA", Journal of Molecular Evolution, (1997) 45: 161–167.

Richard J. Lipton, "DNA Solution of Hard Computational Problems", Science, vol. 268, Apr. 28, 1995, pp. 542–545.

Frank Guarnieri, et al., "Making DNA Add", Science, vol. 273, Jul. 12, 1996, pp. 220–223.

Natalie Milner et al., "Selecting effective antisense reagents on combinatorial oligonucleotide arrays", Nature Biotechnology, vol. 15, Jun. 15, 1997, pp. 537–541.

Ann Caviani Pease et al., "Light-generated oligonucleotide arrays for rapid DNA sequence analysis", Proc. Natl. Acad. Sci. USA, vol. 91, pp. 5022–5026. May 1994.

Glenn H. McGall, et al., "The Efficiency of Light-Directed Synthesis of DNA Arrays on Glass Substrates", Journal of the American Chemical Society, vol. 119, No. 22, Jun. 4, 1997 pp. 5081–5090.

Susan L. Berent et al., "Comparison of Oligonuleotide and Long DNA Fragments as Probes in DNA and RNA Dot, Southern, Northern, Colony and Plaque Hybridizations", BioTechniques, May/Jun. 1985, pp. 208–220.

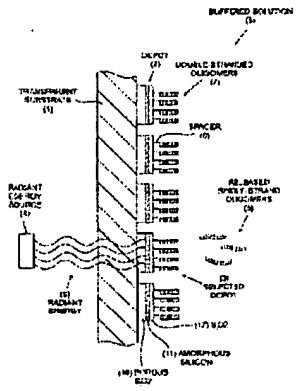
(List continued on next page.)

Primary Examiner—Jezia Riley

[57] ABSTRACT

The present invention is directed to a method for providing oligonucleotides or oligonucleotide analogs having known subunit sequences in which the desired oligomers are released from selected storage sites in one, two, or three dimensions, on a substrate by locally denaturing double-stranded complexes at the storage sites containing the desired oligomers. The released oligomers are useful in schemes for determining solutions to mathematical problems, in methods wherein hybridizing oligomers are used to encrypt and transmit data, in diagnostic and screening assay methodologies, and as primers or building blocks for synthesizing larger polynucleotides.

17 Claims, 6 Drawing Sheets



OTHER PUBLICATIONS

Chad A. Mirkin et al., "A DNA-based method for rationally assembling nanoparticles into macroscopic materials," Nature, vol. 382, Aug. 15, 1996, pp. 607–609.

L.E. Morrisen and L. M. Stols, "Sensitive fluorescence-based thermodynamic and kinetic measurements of DNA hybridization in solution", *Biochemistry* 32 (1993) 3095–3104.

M. Chee, et al., "Accessing genetic information with high-density DNA arrays", Science 274 (1996) 610-614.

M. J. Kozal et al., "Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays", Nature Medicine 2 (1996) 753–759.

D. I. Stimpson et al., "Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides", Proc. Nat Acad. Sci. USA 92 (1995) 6379–6383.

E.L. Sheldon et al., "Matrix DNA hybridization", Clinical Chemistry 39 (1993) 718–719.

Charles R. Cantor and P.R. Schimmel, *Biophysical Chemistry*, Part III (Freeman, New York, 1980) pp. 1217 & 1226–1234.

Ted Kamins, *Polycrystalline Silicon For Integrated Circuit Applications*, Kluwer Academic Publishers, 1988, pp. v–xii and 155–174.

Sambrook, J., et al., *Molecular Cloning, A Laboratory Manual*, from Chapter 11, "Synthetic Oligonucleotide Probes" (Cold Spring Harbor Laboratory Press, 1989), pp. 11.2–11.19, 11.45–11.49, and 11.52–1.61.

Francisco J. Ayala, et al., *Modern Genetics*, Second Edition, from Chapter 9, "DNA Manipulation" (Benjamin/Cummings, Menlo Park, CA, 1984), pp. 262–267 and Appendix 1.

R. J. Britten et al., "Repeated Sequences in DNA", Science, 161 (No. 3841), Aug. 9, 1968, pp. 529–540.

R. J. Britten, et al., "Analysis of Repeated DNA Sequences By Reassociation", Methods In Enzymology, vol. 29, Part E (1974) pp. 363–418.

James G. Wetmur, et al., "Kinetics of Renaturation of DNA", J. Molecular Biology 31 (1968) 349–370.

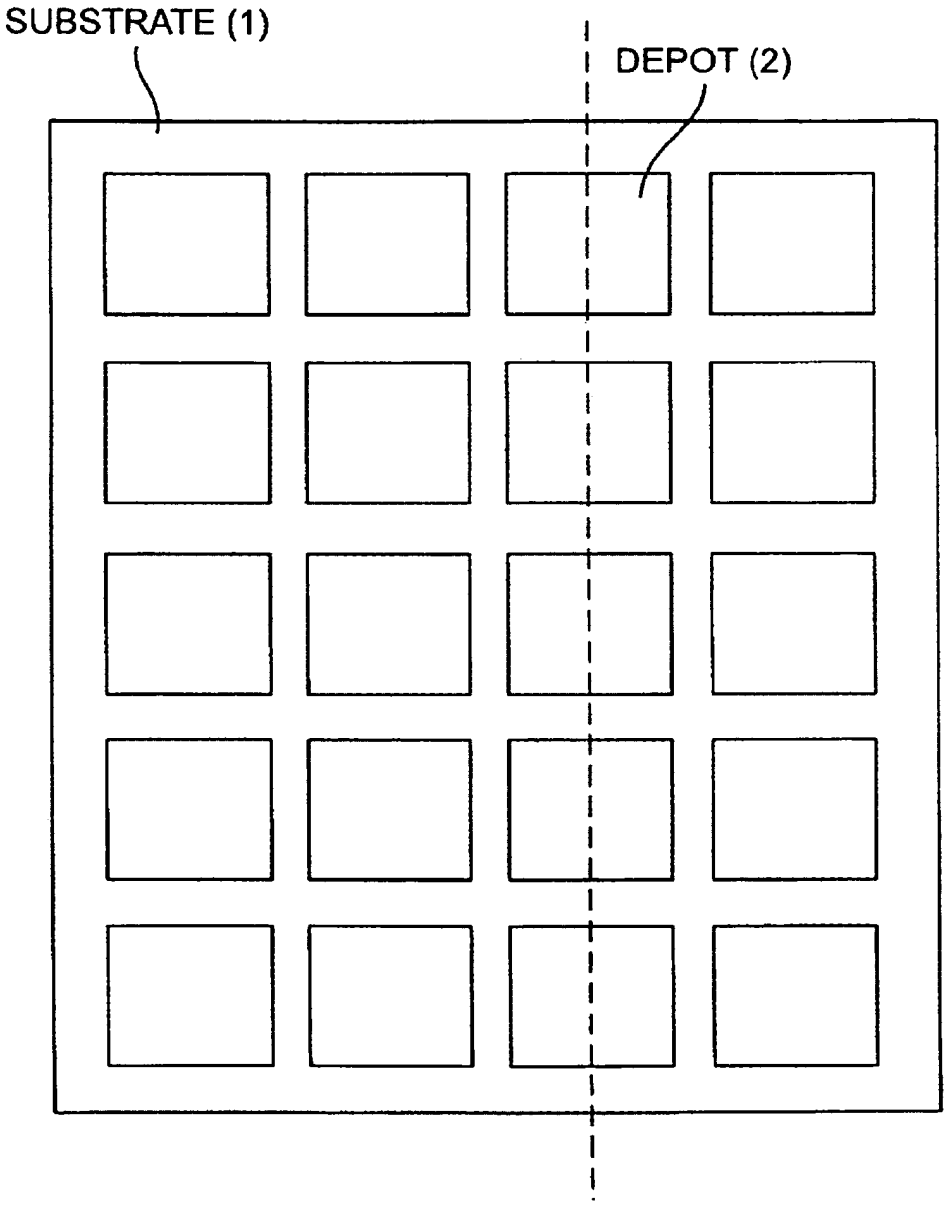


FIG. 1

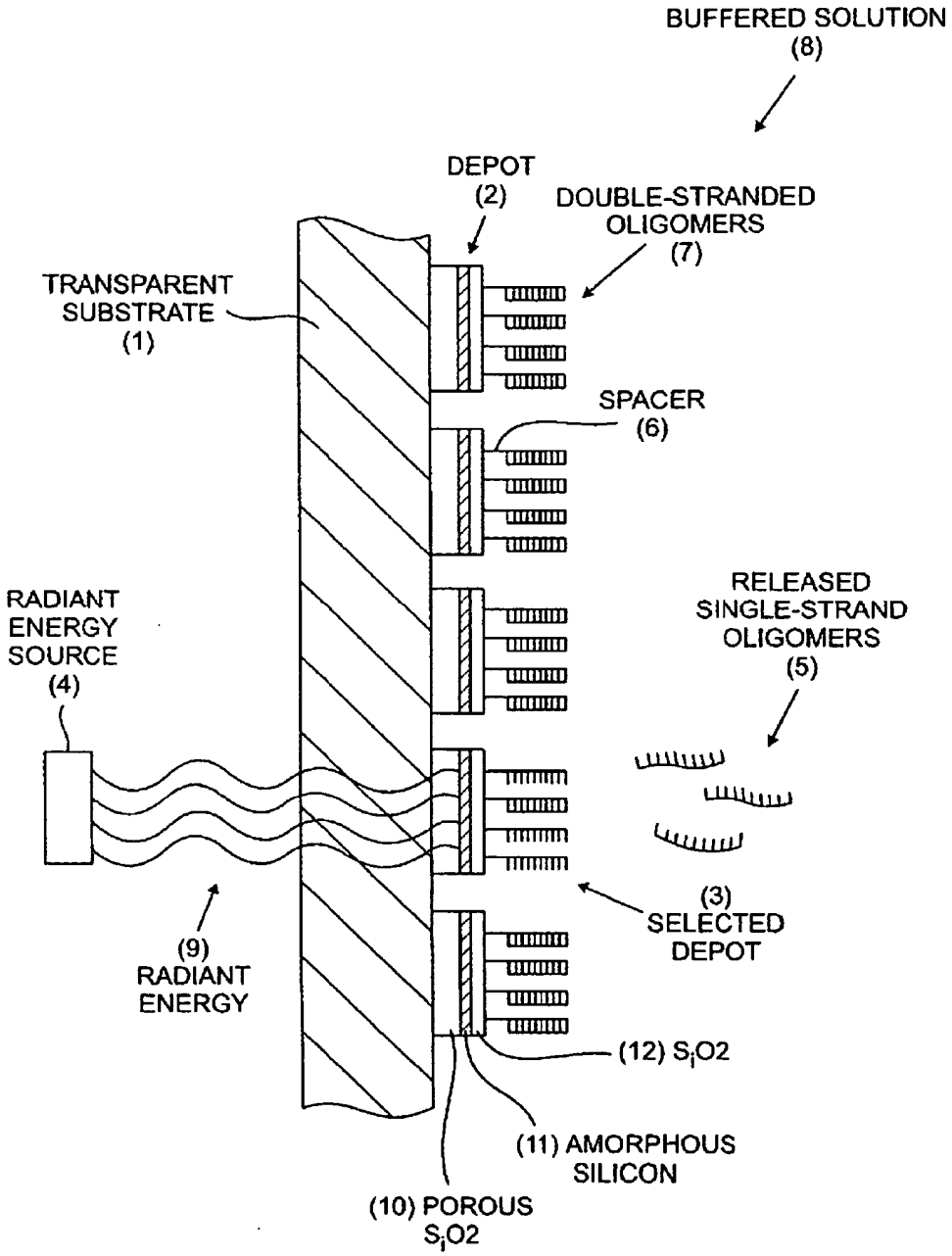

BUFFERED SOLUTION
(8)
DEPOT
(2)
DOUBLE-STRANDED
OLIGOMERS
(7)
TRANSPARENT
SUBSTRATE
(1)
SPACER
(6)
RADIANT
ENERGY
SOURCE
(4)
RELEASED
SINGLE-STRAND
OLIGOMERS
(5)
(3)
SELECTED
DEPOT
(9)
RADIANT
ENERGY
(12) S_iO2
(11) AMORPHOUS
SILICON
(10) POROUS
S_iO2

FIG. 2

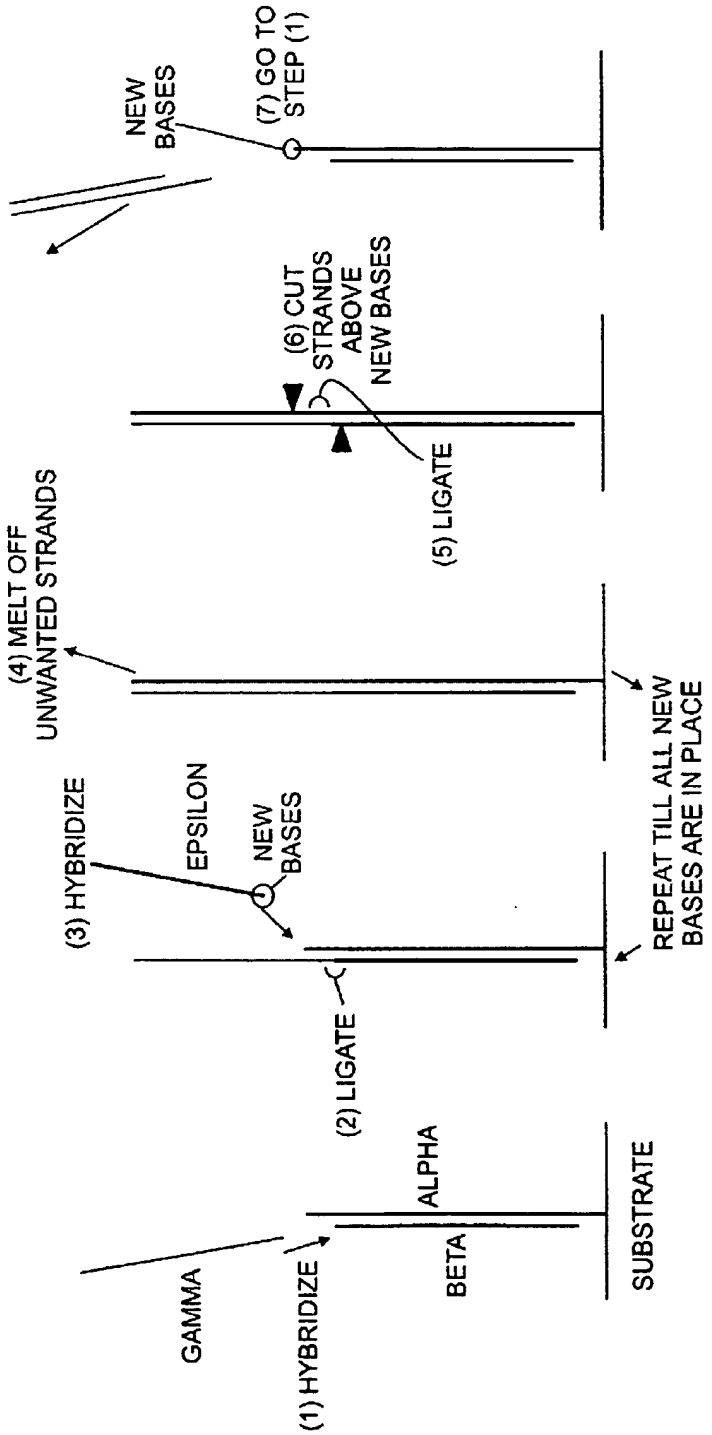

(1) HYBRIDIZE
GAMMA
BETA
ALPHA
SUBSTRATE
(2) LIGATE
(3) HYBRIDIZE
EPSILON
NEW BASES
REPEAT TILL ALL NEW BASES ARE IN PLACE
(4) MELT OFF UNWANTED STRANDS
(5) LIGATE
(6) CUT STRANDS ABOVE NEW BASES
(7) GO TO STEP (1)
NEW BASES

FIG. 3

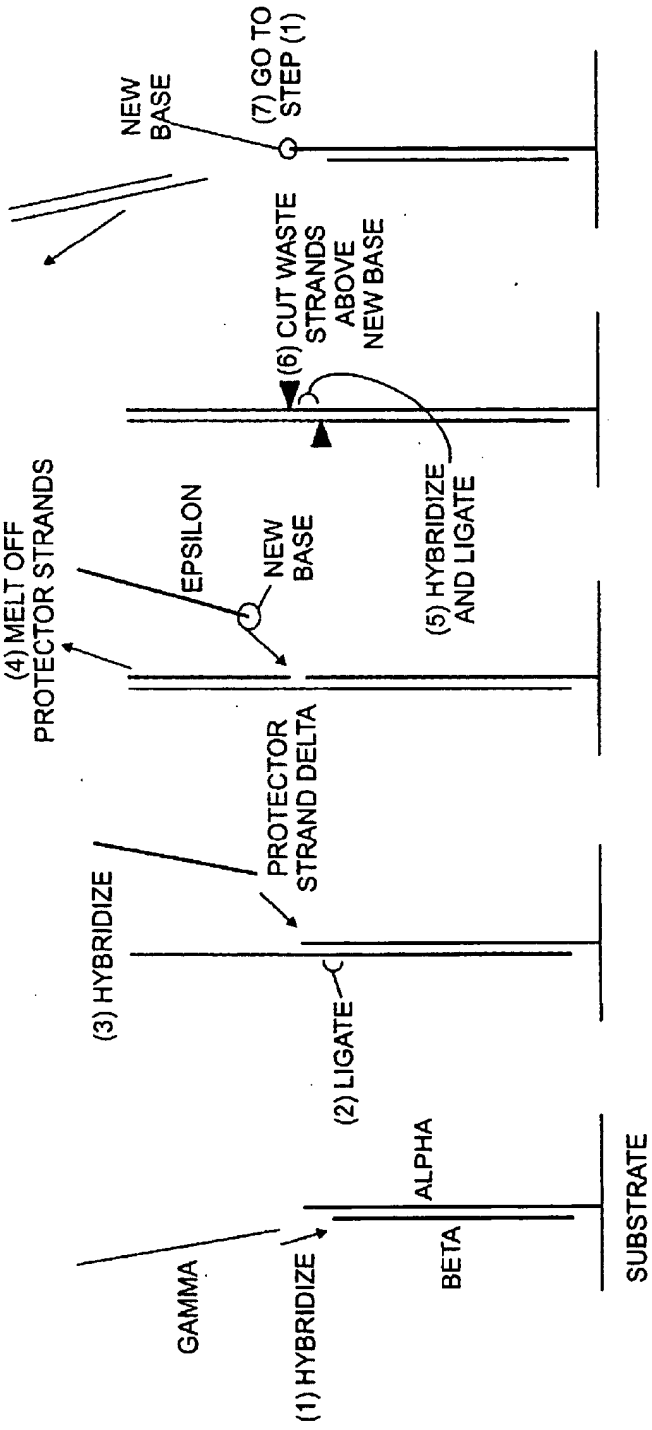

NEW BASE
(7) GO TO STEP (1)
(6) CUT WASTE STRANDS ABOVE NEW BASE
(5) HYBRIDIZE AND LIGATE
(4) MELT OFF PROTECTOR STRANDS
EPSILON
NEW BASE
PROTECTOR STRAND DELTA
(3) HYBRIDIZE
(2) LIGATE
ALPHA
BETA
GAMMA
(1) HYBRIDIZE
SUBSTRATE

FIG. 4

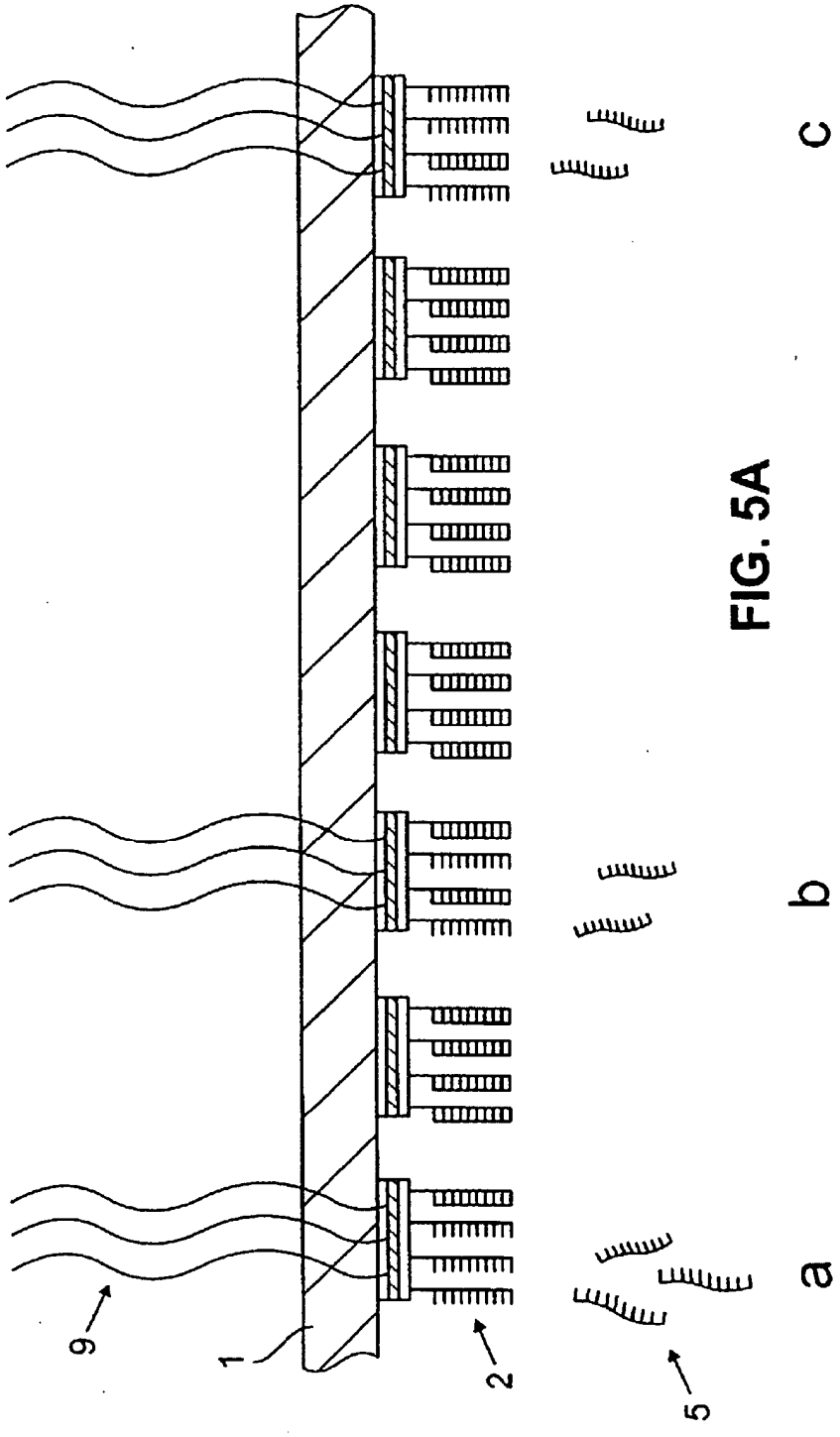


FIG. 5A

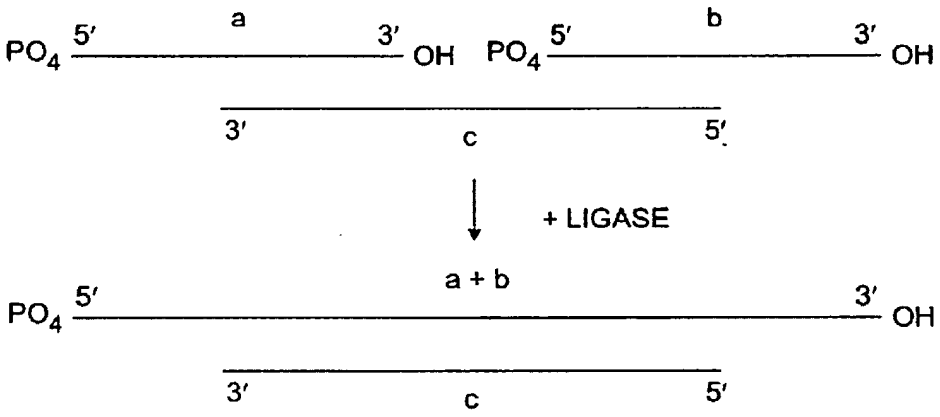


FIG. 5B

METHOD OF GENERATING NUCLEIC ACID OLIGOMERS OF KNOWN COMPOSITION

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. application Ser. No. 09/018,248 filed Feb. 3, 1998.

FIELD OF THE INVENTION

This invention pertains to a method for providing oligomers of known subunit sequence which hybridize specifically to DNA and RNA having complementary nucleotide sequences, in which the desired oligomers are released from selected storage sites on a substrate by locally denaturing double-stranded complexes at the storage sites containing the desired oligomers. The released oligomers are oligonucleotides or oligonucleotide analogs, and are useful in schemes for determining solutions to mathematical problems, in methods wherein hybridizing oligomers are used to encrypt and transmit data, in diagnostic and screening assay methodologies, and as primers or building blocks for synthesizing larger polynucleotides. The present invention also features providing oligomers having desired subunit sequences from a device comprising a substrate supporting an array of oligomer-storing depot sites made by a novel method for the synthesis of DNA arrays which utilizes local melting of hybridized DNA and produces a set of substrate-attached oligomers of known subunit sequence. The present invention has applications in the fields of molecular computation, biochemistry, molecular biology, pharmacology, medical diagnostic technology, and data encryption and transmission.

BACKGROUND OF THE INVENTION

All publications and patent applications herein are incorporated by reference, fully as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Various strategies for finding solutions to mathematical problems have been devised which use sets of DNA oligonucleotides having selected length and sequence properties. For example, DNA-based methods are developed for solving a Hamiltonian path problem (Adleman, Science, 1994, Vol. 266, pages 1021–3), a "satisfaction" problem (Lipton, Science, 1995, Vol. 268, pages 542–5), and for performing addition (Guarnieri et al., 1996, Science, vol. 273, pages 220–223) and matrix multiplication (Oliver, J. Molecular Evolution, 1997, Vol. 45, pages 161–7) of non-negative numbers. Each computation requires a set of oligonucleotides having properties tailored to the problem to be solved. Thus, a rapid and efficient method for providing custom sets of oligonucleotides having selected sequence and length properties is essential for efficient application of DNA-based computation methods.

The present ability to detect oligonucleotides that are bound in a sequence-specific manner to discrete sites of a hybridization array permits the use of oligonucleotides to encrypt and transmit data; a use which, like nucleic acid computation, requires numerous custom sets of oligonucleotides having particular sequences and hybridization properties.

Oligonucleotides are also used as hybridization probes to detect specific nucleic acid sequences in DNA and RNA samples immobilized on a variety of filter and solid supports, as in DNA and RNA Dot, Southern, and Northern

blots, and in colony and plaque hybridization assays. These methodologies are widely used in the isolation and cloning of specific nucleic acids, and the diagnosis of disease caused by pathogens and genetic mutations (Berent et al., BioTechniques, issue of May/June 1985, pages 208–20; and J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, Chapter 11). After detection of labeled probes on a hybridization filter, it is a common practice to expose the hybridization filter to denaturing conditions such as solution of low ionic strength and high temperature, in order to wash the hybridizing probe molecules from the filter, making the filter ready for re-hybridization with a different hybridization probe (Protocols for DNA and RNA Transfer, DNA Electrotransfer, and Protein Transfer to Biodyne A Nylon Membranes, Pall Ultrafine Filtration Corporation, East Hills, N.Y., 1985, page 14).

Sets of oligonucleotides of defined sequence are used as primers for polymerases in polynucleotide synthesis and in nucleic acid amplification, for example, by the polymerase chain reaction (PCR, see Erlich, PCR Technology, Stockton Press, New York, 1989, in entirety). Sets of oligonucleotides of defined sequence are also used as probes of macromolecular structure, and are screened to identify oligomers which, either as antisense or as triplex-forming oligonucleotides, bind specifically to a native target nucleic acid such as a folded mRNA molecule (see, for example, Milner et al., Nature Biotechnology, 1987, Vol. 15, pages 537–41; and U.S. Pat. No. 5,176,996).

More recently, oligonucleotides have been immobilized or synthesized in micro-arrays on solid supports of material such as glass or SiO_2. "DNA chips" produced in this manner are useful for detecting or capturing multiple nucleic acid targets, for determining the nucleic type sequence of a target nucleic acid, for simultaneous analysis of the expression of thousands of genes, large scale gene discovery, DNA polymorphism screening, and mapping of genomic DNA clones, and are well suited for use in medical diagnostic assays for detection of pathogen infection and genetic mutation (for example, see U.S. Pat. No. 5,445,934; U.S. Pat. No. 5,503,980; U.S. Pat. No. 5,605,662; Caviani-Pease et al., 1994, PNAS, Vol. 91, pages 5022–6; and reviews by Ramsay, 1998, Nature Biotechnology, Vol. 16, pages 40–44; and Marshall et al., 1998, Nature Biotechnology, Vol. 16, pages 27–31).

Fodor et al. (U.S. Pat. No. 5,445,934, col. 3–21, 23–32) describes photolithographic solid-phase synthesis of arrays of oligomers, including arrays of oligonucleotides of known nucleotide sequence. The oligomer arrays are synthesized on a substrate by attaching photo-removable groups to the surface of a substrate, exposing selected regions of the substrate to light to activate those regions, and attaching monomeric subunits with photo-removable groups to the activated regions. The steps of photo-activation and attachment can be repeated until oligomers of desired length and sequence are synthesized. According to the current state of the art pertaining to the photolithographic synthesis of polynucleotide arrays, there is only a 92–94% chance that a new nucleotide will be incorporated where desired (McGall et al., J. Am. Chem. Soc., 1997, vol. 119, pages 5081–90). Current technology thus imposes certain constraints on the possible array configuration, such as a practical upper limit on the number of nucleotides of approximately ten.

McGall et al. (U.S. Pat. No. 5,412,087, col. 4–20) describes substrates with surfaces to which are attached compounds having a thiol functional group protected by a

photo-removable protecting group, which compounds can be used to construct arrays of immobilized anti-ligands, such as oligonucleotide probes.

Heller et al. describe a "master" DNA chip comprising a controllable, integrated array of micro-electrodes, and teaches denaturing double-stranded complexes comprising oligonucleotides at selected sites by increasing the negative potential and by use of chemical denaturants, in a process in which the oligomers hybridized at the selected sites are transferred to, or "printed" onto, another chip (U.S. Pat. No. 5,605,662, col. 20, lines 16–39).

DNA oligonucleotides of defined sequence can also be used as structural components of an electronic computer chip (Hollenberg et al., U.S. Pat. No. 5,561,071).

As is apparent from the preceding discussion there are numerous computational, data transmission-related, molecular biological, biochemical, and diagnostic applications which require the use of sets of oligonucleotides or oligonucleotide analogs of defined sequence and length. There currently is a need for a method for rapidly and efficiently providing the various combinations of oligomers required for applications such as those discussed above.

BRIEF SUMMARY OF THE INVENTION

Presented here is a rapid and efficient method for providing a selected set of oligonucleotides and/or oligonucleotide analogs comprising known subunit sequences. The method comprises the steps of

a) obtaining a device for storing and providing oligomers comprising a substrate that supports an array of oligomer depots;

wherein each depot comprises a surface to which are attached a plurality of oligonucleotides and/or oligonucleotide analogs having a selected subunit sequence;

wherein the subunit sequence of the oligomers attached to at least one of said depots is different from the subunit sequence of the oligomers attached to a different depot of said array; and

wherein oligonucleotides and/or oligonucleotide analogs comprising selected subunit sequences are stored at a plurality of depots of said array by being hybridized by Watson-Click pairing to the oligomers attached to the surfaces of said depots to form double-stranded complexes;

b) locally denaturing double-stranded complexes of at least one selected depot of the intact array to release oligomers stored therein, without effecting significant denaturation of double-stranded complexes of the unselected depots of the array; and

c) collecting the oligomers released as a result of locally denaturing double-stranded complexes of said at least one selected depot.

The substrate that supports the array of oligomer-storing depots can be flexible, e.g., a nylon filter, or it can be of a rigid material such as SiO_2 in a DNA chip.

The array of depot sites may consist of from 2 to 10^7 delimited areas wherein as many different types of oligomers are stored. The diameter of the area of each oligomer depot surface to which oligomers are attached can range from about 1 micron to 1 centimeter or more. Using known methods and currently available technology, one skilled in the art can readily fabricate an array of depot sites which are 5–10 microns in diameter, in which array the array density is about 10^6 depot sites per cm^2.

Oligomers comprising a selected subunit sequence can be attached at a depot site directly to the area of substrate surface delimited by the depot boundaries, or they may be attached to the surface of a separate layer of material that is, in turn, attached to the substrate surface at the depot site.

Oligomers are attached to their respective depot sites using protocols known by those skilled in the art for attaching oligomers to a substrate so that the attached oligomers are able to hybridize efficiently with nucleic acids comprising a complementary nucleotide sequence.

The oligomers of known sequence attached to the array of depot sites can be synthesized by methods for synthesizing oligonucleotides and oligonucleotide analogs which are known to those skilled in the art. For example, they can be synthesized in situ on the supporting substrate, e.g. by photolithographic methods, or they can be pre-synthesized and deposited at the depot site, e.g. by micropipette, for chemical attachment.

The present invention also features a method wherein the oligomers of known subunit sequence that are attached to the array of depot sites are synthesized by a novel method which uses local melting of hybridized DNA, DNA ligase, and a restriction enzyme.

In all of the procedures involved in storing and releasing selected oligomers according to the present invention, the depot surfaces to which oligomers are attached are immersed in, or in contact with, buffered solutions of composition suitable for the biochemical or molecular biological operations being carried out.

Depot sites within the array are thermally insulated and/or physically separated from each other so that denaturation of double-stranded oligomer complexes at the selected depots does not cause denaturation of double-stranded complexes at the non-selected depots.

A collection of soluble oligomers of known composition is obtained by locally denaturing double-stranded complexes of the depots of the intact array comprising the desired oligomers, to yield the desired single-stranded oligomers in quantity related to the time and extent of the denaturing treatment. The oligomers are then collected in the buffer solution in which the array is immersed, for use in whatever application is contemplated.

A storage device comprising 10^6 storage depot sites is able to store every possible oligomer 10-mer sequence (4^{10} is approximately equal to 1.0×10^6). Using a storage device comprising about 10^6 depot sites and storing every possible 10-mer, it is possible, with the present invention, to rapidly provide primers or hybridization probes that are complimentary to sites in any target nucleic acid. Also using such a storage device according to the present invention, a primer or hybridization probe of length greater than 10 subunits can be obtained rapidly by providing a set of oligomers of selected subunit sequence which, when ligated end-to-end, produce the desired longer polynucleotide.

Suitable applications for which oligomers can be provided according to the present invention include, but are not limited to, nucleic acid computation, nucleic acid amplification, polynucleotide synthesis by primer extension or by ligating oligonucleotides together end-to end, nucleic acid hybridization for detection or isolation of a target nucleic acid, and data encryption and transmission.

The present invention offers the advantages of rapidly and efficiently providing diverse, custom sets of oligomers, as needed, from a compact and easily accessed storage device. The invention is particularly advantageous for nucleic acid computation, and for encryption and transmission of data in the form of selected sets of hybridizing oligomers, since numerous sets of different oligomers having particular length and sequence properties are needed for both of these

technologies, and these can be provided with facility by the present invention.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 schematically represents an oligomer-storing device comprising a substrate (1) supporting an array of oligomer depot sites (2). The dotted line indicates the section giving the view shown in FIG. 2.

FIG. 2 schematically represents a cross-section through a row of depot sites of the oligomer-storing device shown in FIG. 1, for which the substrate (1) is a transparent substrate. A preferred embodiment of the present invention is shown wherein localized heating of a selected depot (3) is achieved by using a source of radiant energy (4) to irradiate the selected depot site through the transparent substrate (1) to release the desired single-stranded oligomers (5).

FIG. 3 schematically illustrates the seven basic steps of the disclosed ligation/restriction-based method for synthesizing an oligonucleotide array in which ε strands are hybridized to γ strands in step 3. The steps are described in detail below.

FIG. 4 schematically illustrates an alternative method for ligation/restriction-based synthesis of an oligomer array wherein protective δ strands are hybridized to the γ strands in those portions of the DNA-covered substrate where nucleotide addition is not desired.

FIGS. 5A and 5B schematically illustrate a method in which selected DNA oligonucleotides are released from an oligomer storage device (FIG. 5A), and are ligated together to make a longer DNA molecule (FIG. 5B).

DETAILED DESCRIPTION OF THE INVENTION

This invention features methods wherein custom sets of oligonucleotides and/or oligonucleotide analogs having selected subunit sequences are rapidly and efficiently provided by their controlled release from depot sites of an oligomer storage device.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, biochemistry, molecular biology, recombinant DNA, and medical diagnostic technology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Books 1–3, Cold Spring Harbor Laboratory Press; B. Roe, J. Crabtree, and A. Kahn, 1996, DNA Isolation and Sequencing: Essential Techniques, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, In Situ Hybridization: Principles and Practice; Oxford University Press; M. J. Gait (Editor), 1984, Oligonucleotide Synthesis: A Practical Approach, IRL Press; and, D. M. J. Lilley and J. E. Dahlberg, 1992, Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA Methods in Enzymology, Academic Press. Each of these general texts are herein incorporated by reference.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

Nucleic Acid Oligomers

The brief summary of DNA and RNA which follows is not meant to be exhaustive of the subject, but merely to provide a general framework for understanding the present invention. A more complete description of DNA and RNA technology is available in a number of texts, including: J. D. Wilson, M. Gilman, J. Witkowski, and M. Zoller, 1992, "Recombinant DNA", Second Edition, Scientific American Books; and, B. Lewin, 1997, "Genes VI", Oxford University Press. Each of these general texts are herein incorporated by reference.

As set forth above, the present invention relates to nucleic acid biochemistry and molecular biology. Genetic information is stored, transmitted, and expressed by nucleic acids, DNA and RNA, which are constructed of nucleotide subunits. In general, oligonucleotides are linear sequences of a few nucleotides (the Greek-derived prefix oligo- indicates "a few"), while linear sequences of many nucleotides are called polynucleotides (the Greek-derived prefix poly- indicates "many"). The choice of whether to refer to a nucleic acid of a given number of nucleotide subunits as a polynucleotide or as an oligonucleotide is arbitrary. Oligomers are linear sequences of relatively few subunits. A number followed by the suffix -mer refers to an oligomer of the indicated number of nucleotide subunits. For example, an oligomer that contains 12 or 17 bases is referred to as a 12-mer or as a 17-mer, respectively. Each nucleotide contains a phosphate group, a sugar moiety, and either a purine or pyrimidine base. The sugar of DNA is deoxyribose while the sugar of RNA is ribose. Nucleosides consist of a purine or pyrimidine base attached to ribose or deoxyribose. Polynucleotides and oligonucleotides each consist of a linear sequence of nucleotides of DNA or RNA in which the 3' position of the sugar of one nucleotide is linked through a phosphate group to the 5' position of the sugar on the adjacent nucleotide. Ligation is the formation of the phosphodiester bond which joins the adjacent nucleotides in the same nucleic acid chain. Two purine bases and two pyrimidine bases are found in both DNA and RNA. The purines adenine (A) and guanine (G) and the pyrimidine cytosine (C) occur in both DNA and RNA. However, thymine (T) only occurs in DNA and uracil (U) only occurs in RNA. The nucleotides of DNA are deoxyadenylic acid, thymidylic acid, deoxyguanilic acid, and deoxycytidylic acid, while the corresponding nucleotides of RNA are adenylic acid, uridylic acid, guanylic acid, and cytidylic acid. The sugar-phosphate backbones are on the outside of the DNA molecule and the purine and pyrimidine bases are on the inside, oriented in such a way that they can form hydrogen bonds to bases on opposing chains. Adenine (A) can pair only with thymine (T), while guanine (G) can bond only with cytosine (C). Hybridization is the process by which two complementary RNA and DNA strands pair to produce an RNA-DNA hybrid, or by which two complementary DNA single strands pair to produce a DNA—DNA hybrid, also known as double-stranded DNA. Universal base analogues or universal nucleotides are capable of hybridizing with any one of the four DNA nucleotides (Nichols et al., Nature, 1994, Vol. 369, pages 492–3; and Loakes et al., Nucleic Acids Research, 1994, Vol. 22, pages 4039–43). An example of a universal base analogue is 5-Nitroindole (Loakes et al., Nucleic Acids Research, 1994, vol. 22, pages 4039–43).

As used herein, the term oligomers refers to RNA or DNA oligonucleotides, RNA or DNA oligonucleotide analogs, or a combination of RNA and/or DNA oligonucleotides and RNA and/or DNA oligonucleotide analogs, which can be attached to the storage device depot sites, or which can be stored by being hybridized to oligomers attached to the depot sites.

Depending on the purposes for which the oligomers are to be used, the RNA or DNA oligonucleotide analogs can be

oligomers in which from one to all nucleotide subunits are replaced with a nucleotide analog to confer desired properties such as detectability, increased hybridization affinity, resistance to degradation by nucleases, or the ability to covalently modify a target nucleic acid. Such oligonucleotide analogs include but are not limited to oligomers comprising 2'-O-alkyl ribonucleotides, phosphorothioate or methylphosphonate internucleotide linkages, peptide nucleic acid subunits (see U.S. Pat. No. 5,714,331, in entirety), and nucleotides modified by attachment of radioactive, or fluorescent groups, groups which intercalate, cross-link or cleave a nucleic acid, or groups which alter the electronegativity or hydrophobicity of the oligomers. Methods for making and using oligonucleotides and oligonucleotide analogs such as those listed above are well known to those skilled in the art of making and using sequence-specific hybridizing oligomers.

The sizes of the oligomers attached to the depot site surfaces, and of the oligomers stored at the depots, can range from about 4 subunits to 1000 or more subunits in length. The stored oligomers can be longer, shorter, or the same length as the attached oligomers. Oligomers having different lengths, and oligonucleotide analogs having different chemical structures and properties, can be stored in different depots of the same array. Those skilled in the art appreciate that oligomer hybridization specificity and affinity are determined, in part, by the length and chemical structure of the oligomer, and are able to select the structural parameters of the oligomers attached to, and stored in, the depots of the oligomer-storing device that are appropriate for their intended use. For example, the subunit sequences of the attached and stored oligomers can be selected so that they do not comprise self-complementary sequences that stabilize folding of said oligomers into hairpin structures which interfere with formation of inter-strand duplexes. Additionally, the subunit sequences of the attached and stored oligomers can be selected so that the melting temperatures (Tm) of the double-stranded complexes formed by hybridization of the complementary portions of the attached and stored oligomers at all of the depots of the array are within a selected range, e.g., in the range of a selected Tm plus or minus about 5 degrees C., for more efficient control of oligomer storage and release.

The Oligomer Storage Device

A central feature of the present invention is that the desired set of oligomers is provided from an oligomer storage device comprising a substrate (for example, see (1) in FIGS. 1 and 2) supporting an array of oligomer-storing sites, referred to herein as depots. The substrate can have a flat surface that supports the array, or it can be distributed in three dimensions, such as in a gel, a fibrous or granular matrix, or in a porous solid. By depot is meant a site at which oligomers are stored comprising a delimited area or volume that is part of or attached to the supporting substrate, to which are attached hybridizing oligomers comprising a selected subunit sequence (for example, see (2) in FIGS. 1 and 2). A depot site can have any size, shape, or volume, consistent with the objective of the invention of storing and selectively releasing oligomers as needed. By array is meant an arrangement of locations in or on the oligomer-storing device. The locations can be arranged in 2- or 3- dimensional arrays, or other matrix formats. FIG. 1 shows a 2-dimensional 4×5 array of depots on a supporting substrate. The number of locations in the array can range from 2 to 10^7 or more. It is within the knowledge of those skilled in the art to fabricate a rigid substrate supporting an array of oligomer depot sites that can range in diameter from about 1 micron to 1 centimeter or more (see U.S. Pat. No. 5,412,087, col. 8, lines 50–68; U.S. Pat. No. 5,445,934, col. 9, lines 10–18; and Ramsay, Nature Biotechnology, vol. 16, p. 40, 1998). All of the depot sites of a given array can have the same diameter, or a single depot array can comprise depot sites having different diameters. The preferred method of the present invention features storing about 10^2 to 10^7 different types of oligomers of about 8 to 30 subunits in length in a micro-array of thermally isolated depot sites on a rigid substrate.

A substrate which is suitable for supporting immobilized nucleic acids for hybridization analysis can, in general, be adapted for use as an oligomer storage device of the present invention. Accordingly, a variety of different designs and materials are available for preparing the oligomer storage device of the present invention. For example, the storage device may be a flexible filter, e.g., of nylon or nitrocellulose, or it may be of a rigid material such as silica, silicon, glass, crystalline Al_2O_3 ("synthetic sapphire"), beryllium oxide, or a solid substrate coated with a noble metal such as gold. Methods for making such substrate supports for hybridizing oligomers are well known to those skilled in the art. (See U.S. Pat. No. 5,412,087, col. 6, lines 1–39; U.S. Pat. No. 5,445,934, col. 11, lines 49–63; Ramsay, Nature Biotechnology, vol. 16, pages 40–41; Dunanac et al., Genomics, 1989, vol. 4, pages 114–128; Mirkin et al., Nature, vol. 382, pages 607–609, 1996; R. Corn, DNA Computing Overview, last modified Mar. 13, 1998, <http://www.corninfo.chem.wisc.edu/writings/DNAoverview.html>).

The oligomers attached at the depot sites can be attached directly to the surface of the substrate, or to the surface of a pad or pedestal-like structure that is in itself attached to the substrate, which pad or pedestal-like structure can be of material that is the same or different from that of the substrate. FIG. 2 shows oligomers attached to a depot site (2) comprising a pad comprising three different layers ((10), (11), and (12)) affixed to a rigid transparent substrate (1). The depot surface to which the oligomers are attached can be located on a raised feature or in a well-like depression on the surface of the supporting substrate.

Methods for making arrays comprising oligomers attached to depot sites to produce oligomer-storing devices for the present invention are well known. Such methods include in situ synthesis of oligomers attached at their 3' ends to a functionalized surface such glass, SiO_2, or GaAs (for example, see U.S. Pat. No. 5,445,934, col. 23, line 3, to col. 25, line 18; U.S. Pat. No. 5,412,087, col. 4, line 67 to col. 10, line 35; U.S. Pat. No. 5,605,662, col. 17, lines 21–63). Alternatively, pre-synthesized oligomers can be chemically attached to the substrate, e.g., by derivatizing the oligomers or the attachment surface, and then depositing microdroplets of the oligomers at the appropriate depot sites and allowing the oligomers to react with the depot site surface, or by attaching biotinylated oligomers to a streptavidin-coded surface (see U.S. Pat. No. 5,503,980, col. 13, lines 2–9; U.S. Pat. No. 5,412,087, col. 1, line 18 to col. 3, line 13 and col. 6, line 21 to col. 10, line 35; Marshall et al., Nature Biotechnology, vol. 16, pages 27–29, 1998; and Mirkin et al., Nature, vol. 382, pages 607–609, 1996).

A preferred mode of attachment of oligomers to depot surfaces for use according to the present invention is to use uncharged spacer groups ((6) in FIG. 2) to tether the oligomers to the depot surface (U.S. Pat. No. 5,445,934, col. 11, line 49, to col. 13, line 45; Caviani-Pease et al., P.N.A.S., 1994, vol. 91, pages 5022–24), as the use of such spacer

groups is known to increase hybridization efficiency (Marshall et al., Nature Biotechnology, 1998, vol. 16, page 29).

Enzymatic Synthesis of Oligomers in situ

An additional and novel method for making a substrate-supported array of oligomer depot sites which can be used as an oligomer-storing device for the present invention is described as follows. A double-stranded DNA consisting of an α strand and a complementary β strand, denoted α-β (alpha-beta), is synthesized by a known method of oligonucleotide synthesis (see M. J. Gait (Editor), 1984, Oligonucleotide Synthesis: A Practical Approach, IRL Press). One to four or more unpaired nucleotides at the phosphorylated 5' end of the α strand extend beyond the 3'-hydroxyl-terminated end of the complementary β strand as a single-stranded structure that is referred to as a "sticky end," because it can hybridize to another single-stranded nucleic acid having a complementary nucleotide sequence. The sticky 5' ends of the α strands are the sites where new nucleotides are added to the desired oligonucleotides being synthesized. Alternatively, the orientation of the strands of the duplex α-β oligomer with respect to the sticky end may be reversed, although suitable restriction enzymes needed in the nucleotide addition step discussed below are more rare in this case. New nucleotides may be added to the duplex α-β oligomers in a reaction in which the α-β oligomers are free in solution, or are attached to a substrate, as shown in FIGS. 3 and 4.

In one embodiment, a substrate is uniformly covered with duplex α-β oligomers, the DNA-covered surface is divided into local regions referred to as depots, and a different oligonucleotide sequence is synthesized in each depot. The duplex α-β DNA molecules are synthesized and attached to the substrate using known protocols; for example, α oligomers can be synthesized in situ on the substrate by a photolithographic method, and β oligomers can be synthesized by routine chemical methods and hybridized to the attached α oligomers; pre-fabricated α-β DNA molecules can be covalently attached to functionalized substrate SiO groups, biotinylated DNA oligomers can be bound to a streptavidin-coated surface, or thiolated DNA oligomers can be linked to a gold substrate, as discussed above. It is preferred that the 3' end of the α strand of the duplex α-β DNA oligomer be anchored to the substrate through an uncharged spacer group; however, the orientation of the strands of the duplex α-β oligomer with respect to the substrate may be reversed, although suitable restriction enzymes needed in step 6 below are more rare in this case, as noted above.

Synthesis of a different oligonucleotide sequence in each depot is achieved by a sequential series of hybridization, ligation, melting, and cleaving reaction, in which each depot is locally heated in turn so that ε (epsilon) DNA strands comprising the new nucleotides to be added hybridize only to DNA strands of the depot where addition is to occur. Localized heating of the DNA oligomers of the claimed invention may be achieved by any suitable means in accord with the types of oligonucleotides being synthesized, the type of substrate used, and the embodiment of the invention being employed. Suitable methods for locally heating depot sites are discussed in detail below. The temperature for heating is selected, with consideration to the lengths and sequences of the oligomers and to the ionic strength of the reaction solution, to rapidly melt off undesired DNA strands bound to the γ strands without melting the α-β duplex structures, so that the desired ε strands with the nucleotides to be added can hybridize to the exposed γ strands.

The synthesis of DNA strands according to the invention is illustrated as follows, referring to FIGS. 3 and 4 in embodiments in which the duplex α-β oligomers are attached to and uniformly cover a substrate. One possible substrate is comprised of a wafer of Si covered by (1) a thermally-insulating 1 μm thick layer of SiO_2, (2) a heat absorbing 0.5 μm thick layer of amorphous Si and (3) a 0.5 μm thick layer of SiO_2 upon which to anchor the DNA oligomers (see elements (10), (11), and (12), respectively, in FIG. 2). The substrate may be patterned into 10 μm×10 μm pads to better define and thermally isolate the identifiable areas (depots) of the plate. The α strand of the duplex α-β DNA oligomer is anchored to the substrate at its 3' end, and one to four or more unpaired nucleotides at its phosphorylated 5' end extend beyond the 3'-hydroxyl-terminated end of the complementary β strand to form a sticky end. In a preferred embodiment, the Si substrate is replaced with a substrate of transparent crystalline Al_2O_3 to allow back illumination of the desired depots, thus protecting the DNA from direct exposure to the laser radiation. Steps of hybridization, ligation, heating to melt desired portions of the duplex DNA complexes, and cleavage by restriction enzyme, are carried out in suitable buffered solutions for these reactions which are well known to those skilled in the art (see Sambrook et al. and the other previously cited references teaching biochemical and molecular biological methodology). In embodiments in which DNA molecules are synthesized on a substrate, the DNA-covered substrate is immersed in suitable buffer during each reaction step of the method.

Step (1): A set of single-stranded γ (gamma) DNA oligomers is prepared having phosphorylated 5' ends, and in which the nucleotide sequences at the 5' ends are randomly varied so that individual members of the set of γ strands can hybridize with every possible a strand sticky end. The set of γ oligomers is allowed to hybridize with the 5' sticky ends of the α strands. The bases of a number p of nucleotides in each γ strand adjacent to the randomized 5'-terminal nucleotides are universal bases, where p is the number of new nucleotides to be transferred from the ε strands to the ends of the α strands. In theory, p can range in value from 1 to as large a number as desired. In using the invention to make a set of long oligonucleotides which differ from each other at only one or a few nucleotides, it may be practical to use oligomers having large p, so as to add large blocks of nucleotides to the ends of the α strands in a single step. In using the invention to make an array of highly variable oligonucleotides, the upper value of p is limited by the practical need to repeat the steps for adding p nucleotides up to 4^p different times for each set of p nucleotides that are added.

Step (2): In the presence of T4 DNA ligase and ATP, the 5' ends of the γ strands become ligated to the 3' ends of the β strands.

Step (3): Desired strands ε (epsilon) are introduced to hybridize to the γ strands, wherein the ε strands have p nucleotides at their 3' ends which are to be added to the α strands. Since there are 4^p different types of ε strands, p being the number of bases added in each step, with each different ε strand ending in one of the 4^p possible sets of p bases, this step would need to be repeated once for each of the different p-tuples of bases added to the entire substrate or plate, prior to ligating. The ε strands hybridize with the γ strands, with the p bases to be added pairing with the p universal bases on the γ strands. The overall lengths and nucleotide sequences of the γ and ε oligomers are selected so that γ and unligated ε strands form a duplex structure that

melts at a temperature at which the α-β duplexes remain intact. A preferred configuration for the α-β-γ-ε complex is one wherein the 5' ends of ε strands and 3' ends of γ strands form blunt ends.

Step (4): In the case where a single type of oligonucleotide is being made, nicks between the 3' hydroxyl terminations of the ε strands and the 5' phosphate terminations of the α strands are ligated according to Step 5 below.

In the case where an array of different substrate-bound oligonucleotides is being synthesized, with new nucleotides also being added to the ends of DNA oligonucleotides at other locations on the substrate, the undesired ε strands are removed by local heating without melting the α-β duplex portions, for example, by using laser illumination patterned with a lithographic mask, and are washed away. Desired ε strands are then hybridized to exposed γ strands of substrate-bound DNA molecules at the heated locations, by repeating Step 3. Steps 4 and 3 of heating to selectively remove undesired ε strands, and then hybridizing desired ε strands at each location where nucleotides are to be added, are repeated until all locations where nucleotides to be added to the sticky ends of the substrate-bound DNA have been treated.

Step (5): After all desired ε strands are hybridized to the growing DNA molecules, nicks between the 3' hydroxyl terminations of the ε strands and the 5' phosphate terminations of the α strands are ligated using T4 DNA ligase again.

Step (6): The resulting double-stranded DNA molecules are cut with a restriction enzyme that leaves a new sticky end similar to the original α-β sticky end, except that cleavage results in addition of p new nucleotides to the 5' end of the α strand. Cleavage may also result in addition of one or more paired nucleotides to the 3' end of the β strand. In the preferred method, the restriction enzyme that is used is one that cuts at a site adjacent to, but outside of, its specific recognition sequence that is built into the ε-γ sequence, to leave the new sticky end on the growing double-stranded oligonucleotide. An example of such a restriction enzyme which is suitable for use in the invention is Alw 26 I. Restriction enzyme recognition sites in the growing α-β duplex can be protected from unwanted cleavage by methylation of one or both strands at the enzyme recognition site in the α-β duplex to be protected, using the appropriate methylase enzymes, or by incorporation of a methylated nucleotide or a restriction-enzyme-inhibiting nucleotide analog, which incorporation could be carried out during synthesis of the original α-β duplex stem, or in the step wherein new bases are added to the growing duplex DNA molecule.

Step (7): The process is repeated for each new set of bases to be added to the growing duplex DNA molecules.

One skilled in the art can readily design the original α and β oligomers to comprise a recognition site for a restriction enzyme that is different from the one used in the synthetic reactions, so that the polymers can be released after synthesis, if desired.

An alternate and less-preferred procedure is illustrated in FIG. 4 in which steps 3–5 are modified to include use of protective δ strands as follows:

Step (3, modified): An excess of δ (delta) protector strands are prepared which are perfectly complementary to all of the nucleotides of the single-stranded portion of the γ strands extending from the α-β duplex, except that the δ strands comprise 3'-phosphate-terminated ends, or they lack a complementary nucleotide at their 3' ends, so that unwanted ligation of the 3' ends of the δ strands to the 5'-ends of the α strands is prevented. The excess of δ protector strands are introduced to hybridize to and protect the γ strands in non-reacting depots from binding to nucleotide-adding ε strands.

Step (4, modified): In desired locations, the protector δ strands are melted off the γ strands by local heating, for example, by using laser illumination patterned with a lithographic mask, and are washed away. Desired strands ε (epsilon) are then introduced to hybridize to the single-stranded γ oligomers. The remaining steps of the alternate method are as described above for the method in which protector δ strands are not used. If one wants to make some of the strands shorter than normal, so that the δ strands need to be left in place during the restriction step, the strands containing a δ may be protected from cutting by methylation of the restriction enzyme recognition site on the δ strand.

The fidelity of synthesis attained using the above-described method for oligomer synthesis of the present invention permits efficient and accurate synthesis of oligonucleotides in substrate-bound arrays that are considerably longer than those that can be accurately made using current technologies; for example, substrate-bound oligonucleotides of up to 20, 30, 50, or even 100 or more nucleotide subunits, can be accurately made by the present invention.

Storing Oligomers in Depots

Oligomers are stored in the depot array of the storage device by allowing them to hybridize specifically to oligomers comprising complementary subunit sequences which are attached at the depot sites ((2) in FIG. 2), to form double-stranded oligomer complexes attached to the depot sites ((7) in FIG. 2). Those skilled in the art recognize that the number of consecutive complementary nucleotides that must be present in an oligonucleotide so that it hybridizes specifically to a target nucleic acid molecule can vary considerably, from about 4 up to 14 or more, depending on such factors as the complexity of the set of target nucleic acids and the physical conditions (ionic strength, temperature, anionic and cationic reagents, etc.) used in the hybridization and wash steps. The statement that a soluble oligomer hybridizes specifically to a substrate-bound oligomer or other target nucleic acid is intended to mean that a portion of the oligomer comprising a nucleotide sequence complementary to a sequence in the substrate-bound oligomer or other target nucleic acid binds by Watson-Crick base-pairing to the complementary portion of the substrate-bound oligomer or other target nucleic acid to form a stable double-stranded complex, under hybridization conditions that are sufficiently stringent that oligomer molecules having fewer bases complementary to, or forming less stable duplex structures with, said substrate-bound oligomers or other target nucleic acids do not hybridize to said substrate-bound oligomers or other target nucleic acids and form stable double-stranded complexes. Selection of parameters such as the lengths of the complementary portions of the soluble and substrate-bound oligomers and the conditions used in hybridization and wash steps, so that the soluble oligomers hybridize specifically to their substrate-bound counterparts, is well within the capabilities of a person of ordinary skill in the art (e.g., see Sambrook et al., 1989, supra, Chapter 11).

For example, a complete set of oligonucleotides comprising every possible sequence of n consecutive nucleotide subunits can be stored in an array of 4^n depot sites comprising complementary oligomers by exposing the array to the soluble oligomers at a temperature about 25° C. below the lowest melting temperature for the set of double-

stranded complexes to be formed, in a suitable buffer containing a high molar concentration of Na^+. The time required to saturate the 4^n depot sizes with the n-mer oligomers is known to be dependent on the concentrations of the oligomers, the temperature, and the concentration of Na^+ ions. If the soluble oligonucleotides are applied at a concentration of 0.5 mole of single nucleotides per liter and the Na^+ concentration is 1 mole per liter, the time for half of the hybridization reaction to be completed is about 4 seconds for n=10, and about 100 days for n=20. (Britten et al., Methods in Enzymology, 1974, vol. 29, part E, pages 363–418; Wetmur et al., J. Molecular Biology, 1989, vol. 31, page 349; Britten et al., Science, 1968, vol. 161, page 529).

Releasing Selected Oligomers

A custom set of soluble oligomers of known composition is obtained by locally denaturing double-stranded complexes of selected depots of the intact array comprising the desired oligomers, and collecting the oligomers released from the selected depots ((5) in FIG. 2) into the buffer solution in which the array is immersed ((8) in FIG. 2). Denaturation of oligomer complexes at selected depots can be achieved by any of the nucleic acid-denaturing treatments known to those skilled in the art of nucleic acid biochemistry. Those skilled in the art appreciate that the melting temperature of a double-stranded oligonucleotide complex is dependent on the length, nucleotide sequence, and chemical structure of the complex, and on the ionic strength and chemical composition of the solvent (see Sambrook et al., 1989, supra, page 11.46).

The preferred method for denaturing double-stranded complexes at the selected depots to release the desired oligomers is by locally heating the selected depots so as to subject the selected depots to a raised temperature under appropriate solution conditions for a period of time sufficient to release the desired oligomers from the selected depots. Localized heating of the selected depot surfaces can be achieved by any suitable means in accord with the structure and size of the supporting substrate, and the size and disposition of the individual depot sites. For example, selected depots can be locally heated by illuminating the surface of the array, in a suitable buffer and at a temperature below the melting point of the oligomer duplexes, with a pattern of focused irradiation from a radiant energy source ((4) and (9) in FIG. 2), e.g. an argon laser, that heats only those depots storing the desired oligomers. The laser can be mounted on a support which provides precise x-y translation control, to permit controlled heating of one depot at a time, in serial fashion. Alternatively, the laser can have a broad beam that can irradiate a mask, the image of which can irradiate all of the depots in the array at once. The mask can thus be used to shield the unselected depots so that only those comprising the desired oligomers are heated. To heat a single depot having a surface area of about 100 μm^2 to about 70° C. in a suitable buffered solution to locally melt double-stranded DNA duplexes stored at the heated depot will require roughly 10 milliwatts of argon laser light (488 nm). Use of a substrate which is transparent to argon laser light, e.g. crystalline Al_2O_3, to support thermally isolated, light-absorbing, depot surfaces to which the oligomers are attached, allows back illumination of the desired depots as shown in FIG. 2, thus protecting the oligomers from direct exposure to the laser radiation. A substrate of Al_2O_3 is also advantageous because the high thermal conductivity of Al_2O_3 permits the substrate to act efficiently as a heat sink, by drawing heat away from the irradiated depot sites and so providing greater thermal isolation of the unselected depot sites. Alternatively, the storage device substrate comprising the depot array could be in contact with, or have integrated within it, a controllable, addressable, array of resistive heating elements which is spatially aligned with the depot array, so that application of current to selected resistive heating elements locally heats selected depots proximal to the activated heating elements to release the desired oligomers. Heller et al. teach fabrication of a silicon substrate into which is integrated a micro-array of electronically addressable micro-locations corresponding to a micro-array of DNA storage sites (U.S. Pat. No. 5,605,662, col. 9–10, 12–16). Accordingly, it is within the knowledge of those skilled in the art of microlithography and thick film circuitry to fabricate a DNA chip in which there is integrated an array of electronically addressable micro-locations comprising resistive heating elements such as can be formed, for example, by depositing undoped polycrystalline silicon at positions between addressable conducting wire grids (Kamins, Polycrystalline Silicon for Integrated Circuit Applications, 1988, Kluwer Academic Publications, Boston). As described by Heller et al., metal contact pads along the outside perimeter of the chip permit wiring such a chip comprising an integrated electronically addressable micro-array to a microprocessor-controlled power supply and interface for controlling the device (U.S. Pat. No. 5,605,662, col. 12). The amounts of oligomers released by localized heating can be controlled by varying the amount of heat applied, e.g., by controlling the intensity of the laser light or the temperature of the resistive heater, and/or by varying the time period during which heat is applied. According to the preferred method, the localized heating of selected depots to release desired oligomers stored therein is electrically controlled by a programmable microprocessor and an interface for controlling the process. By the method of the present invention, local heating of selected depots will cause oligomer duplexes at the heated depots to melt in a short time of the order of seconds, to yield single-stranded oligomers in quantities related to the time and extent of heating.

Heller et al. teach that denaturation of DNA at selected depots can also be induced by locally increasing the negative electric potential at the selected depots (Heller et al., U.S. Pat. 5,605,662, column 20). Thus, an array of microelectrodes integrated within, or closely associated with, a substrate supporting an oligomer-storing array of depot sites can be used to create denaturing conditions at selected depots of the array to practice the present invention. In addition positively charged chaotropic agents and other denaturants can be added to the solution in contact with the selected depots to promote denaturation of the attached double-stranded complexes. Exposure to denaturing solution conditions can be limited to the depots selected for denaturation by surrounding the selected depot surfaces with a liquid-impermeable barrier that prevents the denaturing solution from contacting non-selected depot surfaces. For example, individual depots of a large-scale array, in which depot surfaces are 0.1 to 10 mm or more in diameter, can be situated in wells or surrounded by raised divider walls to be "fluidically isolated" from each other, so that selected depot surfaces can be exposed to denaturing solution without also exposing non-selected depot surfaces to the denaturing conditions. Denaturation of selected depots, whether by localized heating, application of increased negative potential, denaturing solution, or any combination of these means, can be carried out serially, one depot at a time, or in parallel with multiple depots being treated simultaneously.

Collecting and Using the Released Oligomers

Oligomers released from selected depot sites following denaturation of double-stranded complexes at those sites

((5) in FIG. 2) are collecting by collecting the solution in contact with the treated depot surfaces ((8) in FIG. 2). The solution in contact with the oligomer-storing depot array can be enclosed or contained within a reservoir, and once the desired oligomers are released into the solution, it can be collected by any suitable means, e.g. by a manually operated or automated pipetting device, or a syringe. Alternatively, the solution containing the desired oligomers can be removed from the reservoir and transferred to a suitable collecting device, and fresh solution can be added to the reservoir in its place, e.g. to wash away residual oligomers in preparation for releasing a different set of oligomers, by using automated or microprocessor-controlled pumps which direct the flow of the different solutions through tubes connected to the reservoir.

The collected oligomers may then be used in protocols which employ a customized set of oligonucleotides or oligonucleotide analogs. Such protocols include, but are not limited to, protocols for nucleic acid computation, nucleic acid amplification, polynucleotide synthesis by primer extension or by ligating together overlapping complimentary oligonucleotides, nucleic acid hybridization for detection or isolation of a target nucleic acid, and data encryption and transmission.

EXAMPLES

The following examples further demonstrate several preferred embodiments of this invention and are offered by way of illustration, but should not be construed as limiting the claims thereof. Those skilled in the art will recognize numerous equivalents to the specific embodiments described herein. Such equivalents are intended to be within the scope of the claims.

Example 1

Synthesis of a DNA oligonucleotide by the ligation/restriction method

As a concrete example for the case in which a single base is to be added to the strands in each step (i.e., p=1), the following oligomers are selected to carry out the needed reactions:

34-mer, α: 5' TCTTAACATAGGAATTTGAGGCAGTACGCAAAAA 3'-biotin (B) (SEQ ID NO: 1).

30-mer, β': 3' AGAATTGTATCCTTAAACTCCGTCATGCGT 5' (SEQ ID NO: 2).

26-mer, β: 3' TTGTATCCTTAAACTCCGTCATGCGT 5' (SEQ ID NO: 3).

17-mer, γ: 3' TCACGTCAGAGCNNNNN 5' (SEQ ID NO: 4), wherein the first N in the 3'→5' direction is a universal base and the subsequent N's designate A, C, G, or T.

13-mer, ϵ_A: 5' AGTGCAGTCTCGA 3' (SEQ ID NO: 5).

13-mer, ϵ_T: 5' AGTGCAGTCTCGT 3' (SEQ ID NO: 6).

13-mer, ϵ_G: 5' AGTGCAGTCTCGG 3' (SEQ ID NO: 7).

13-mer, ϵ_C: 5' AGTGCAGTCTCGC 3' (SEQ ID NO: 8).

The sequence of oligomer SEQ ID NO: 2 (β') consists of 18 A-T's and 12 G-C's, chosen to minimize the number of A-T and/or G-C matches of the sequence with itself for shifts of up to ±20 bases. It is further chosen to have no more than 3 A's, T's, or G-C's in a row; no more than 2 G's or 2 C's in a row. These selections are to ensure that the strands will not form hairpins. Oligomer SEQ ID NO: 2 (β') is chosen to lack the restriction enzyme Alw 26 I recognition sequence GTCTC/CAGAG or either of the four base pair segments of that sequence. Oligomer SEQ ID NO: 1 (α) is complementary to the full sequence of β', and has in addition a quartet of A's and a biotin group at the 3' end for attaching the α's to the substrate. SEQ ID NO: 3 (β) is identical to β' except that four bases are missing from the 3' end to produce a 4-base sticky end when hybridized to α. In oligomer SEQ ID NO: 4 (γ), the first N in the 3'→5' direction is a universal base, such as 5-Nitroindole, and each of the subsequent N's are random deoxyribonucleotide bases. The concentration of any one particular version of γ will be 1/256 of the total. The ε oligomers (SEQ ID NOs:5–8) each contain one of the two single-stranded sequences from the duplex DNA Alw 26 I restriction enzyme recognition sequence, which cuts leaving the 5' sticky end indicated:

5' . . . NNNGTCTCN 3' (SEQ ID NO: 9, from the ε strand)

3' . . . NNNCAGAGNNNNN 5' (SEQ ID NO: 10, from the γ strand),

wherein the 5'th N from the 5' end of the γ strand is a universal base, and the other Ns designate A, C, G, or T.

The detailed steps in making a DNA hybridization array are as follows.

Step (1). We start by attaching α oligomers uniformly over the substrate, e.g., by using the affinity of biotin for a streptavidin-coated glass surface, and β strands are then hybridized with the anchored a strands, giving:

5' TCTTAACATAGGAATTTGAGGCAGTACG-CAAAAA 3'-B (α, SEQ ID NO:1)

3' TTGTATCCTTAAACTCCGTCATGCGT 5' (β, SEQ ID NO:3).

Step (2). The set of γ DNA strands (SEQ ID NO: 4) is introduced to hybridize with the sticky ends of the α strands, and the ends of the γ DNA strands are ligated to the ends of the β strands of the anchored α-β DNA by incubating with T4 DNA ligase and ATP, giving:

5' TCTTAACATAGGAATTTGAGGCAGTACG-CAAAAA 3'-B (α)

3' TCACGTCAGAGCNNNNNTTGTATCCT-TAAACTCCGTCATGCGT 5' (γ+β)

where the α strand is SEQ ID NO: 1, and the γ+β strand is SEQ ID NO: 11 wherein the first N in the 3'43 5' direction in γ is a universal base and the subsequent N's designate A, C, G, or T.

Step (3). The DNA-covered substrate is incubated in the presence of an oligomer denoted ϵ_X containing the base X to be added to the α strand, so that the ϵ_X oligomers hybridize to the γ strands. In this example, ϵ_A=SEQ ID NO: 5; ϵ_T=SEQ ID NO: 6; ϵ_G=SEQ ID NO: 7; and ϵ_C=SEQ ID NO: 8.

Step (4): To add one of the 4 bases A, T, G, or C, to DNAs of 4 or more different depots, the hybridization step would need to be repeated with each of the 4 different ϵ_X strands at the desired substrate locations prior to ligating and cleaving. After the first step in which an ϵ_X strand is hybridized to the substrate-bound DNA, and before each subsequent ϵ_X addition step, undesired ϵ_X strands are melted away from the DNA of the depots where the nucleotides are to be added by local heating, e.g., by using laser illumination patterned with a lithographic mask for 10 seconds to give a local temperature of approximately 70° C., thereby producing the same duplex DNA structure comprising a duplex α-β portion produced in Step 2, wherein γ DNA strands in the selected areas are receptive to one of the ϵ_X oligomers. Desired ϵ_X strands are then hybridized to exposed γ strands of substrate-bound DNA molecules at the heated locations by repeating Step 3. Local heating to selectively remove undesired ϵ_X strands without melting duplex α-β portions (Step 4), and hybridization of desired ϵ_X strands at each location where

nucleotides are to be added (Step 3), are repeated until all depots where nucleotides are to be added have been treated with a desired ϵ_X oligomer.

Step (5): After all desired ϵ strands are hybridized to the growing DNA molecules, nicks between the 3' hydroxyl terminations of the ϵ strands and the 5' phosphate terminations of the α strands are ligated using T4 DNA ligase again. Ligation of the hybridized ϵ_X strands to the α strands by incubating with T4 DNA ligase and ATP gives:

5' AGTGCAGTCTCGNTCTTAACATAG-GAATTTGAGGCAGTACGCAAAAA 3'-B(ϵ+α)

3' TCACGTCAGAGCNNNNNTTGTATCCT-TAAACTCCGTCATGCGT 5' (γ+β),

where the γ+β strand is SEQ ID NO: 11 as described above, and wherein and the ϵ+α strand is SEQ ID NO: 12 wherein N is A, C, G, or T.

Step (6): The DNA-covered substrate is incubated at 37° C. with Alw 26 I restriction enzyme, and a small sticky-ended double-stranded oligomer is cut off and washed away producing:

5' NTCTTAACATAGGAATTTGAGGCAG-TACGCAAAAA 3'-B (α+N)

3' NTTGTATCCTTAAACTCCGTCATGCGT 5' (β+N),

where α+N is SEQ ID NO: 13 and β+N is SEQ ID NO:14, wherein N is A, C, G, or T, and

5' AGTGCAGTCTCG 3' (SEQ ID NO: 15)

3' TCACGTCAGAGCNNNN 5' (SEQ ID NO: 16),

wherein the first N in the 3'→5' direction is a universal base and the subsequent N's designate A, C, G, or T.

This leaves the new deoxyribonucleotides X of ϵ_X added to the α strands, and the α-β strands in a state precisely like that encountered in step 2, except for being one base pair longer.

Step (7): The synthetic cycle is now repeated by returning to Step 2 of the above-described example. By repeating Steps 2–6 one may now add as many bases as desired in what ever pattern is needed.

Step (8). When the α strands have the desired sequence, the lengthened β strands are melted off and washed away. The β' strands are then allowed to hybridize with the α strands, leaving the newly synthesized oligonucleotides in single-strand form, attached at their 3' ends to the blunt-ended α-β duplexes at the 5' ends of the α strands. If the number of added nucleotides happens to be four, this last step is not needed.

The ϵ strands are more than 50% G-C's and would be expected to dissociate at a rate of less than 10^{-4} s^{-1} or less at 22° C., compared to 10^{2} s^{-1} at 70° C. (from extrapolation of the data in FIG. 6 of Morrison et al., Biochemistry, 1993, vol. 32, pages 3095–3014; see also C. Cantor and P. Schimmel, Biophysical Chemistry, 1980, Freeman Press, New York, page 1217). The melting point for similar 14-mers is about 40° C. (Wallace et al., Nucleic Acid Research, 1979, vol. 6, pages 3543–3557). Thus, a 10 sec heat pulse raising the temperature of a spot to 70° C. will result in a 99.9% chance that a new base is incorporated where it is wanted and a similar chance that it is not incorporated elsewhere if the temperature there is less than 20° C. The β' strands dissociate at a rate of roughly 10^{-5} s^{-1} at 70° C., and thus the structure should be quite stable under the temperature cycling needed for Steps 2–7.

It is estimated that when one depot is heated to the 70° C. required in Step 4 the temperatures of any unilluminated neighboring depots will not rise above 20° C. if the substrate is heat-sunk to near 0° C. Although the dissociation rates for oligomers are a steep function of temperature, the borders of the depots will contain sequences that do not correspond to the programmed growth. In operation, the area between the depots should not be subjected to heating; e.g., through use of a mask.

At concentrations of 10^{-6} M (moles per liter), hybridization reactions rates are of the order of 1 s^{-1}. The rate limiting steps in this scheme are the two ligation steps and the one restriction step. According to their catalog, one New England Biolabs (NEB) unit for the T4 DNA ligase gives 50% ligation of Hind III fragments in 30 m at a 5' DNA termini concentration of 10^{-7} M. Using a high concentration of enzyme will result in sufficiently complete ligation in a few minutes. The restriction enzyme will also act in a few minutes. The ligation and restriction enzyme cleavage steps need to occur only once in the four cycles. One may thus estimate that growth of a DNA array by the method described would take less than 30 minutes per four bases added, comparable to the 1 hour per four bases in the early light-directed synthesis work of Ref. 7.

Example 2

Releasing a set of oligonucleotides of known sequence from an oligomer-storing device

This example, illustrated in FIGS. 5A and 5B, demonstrates an embodiment of the invention in which three selected DNA oligonucleotides, a, b, and c, are released from an oligomer storage device, and oligomers a and b are hybridized end-to-end to complementary oligomer c and are ligated together to produce a longer DNA molecule. This method is useful, for example, as a step in a protocol for solving a Hamiltonian path problem (Adleman et al., Supra, pages 1022–1023), or for making a synthetic gene.

The device that stores and releases the oligomers comprises a 1 cm×1 cm wafer of crystalline Al_2O_3 substrate ((1) in FIG. 5A) that supports a square array of 165×165 depot pads. Wafers of crystalline Al_2O_3, "synthetic sapphire", which are suitable for use with the present invention can be obtained from Saphikon, Milford, N.H., 03055. The top surface of each depot pad is 50 μm×50 μm, and the depot pads are spaced 10 μm apart in both x and y directions in the array. Each depot pad comprises 3 layers, (1) a thermally insulating 1 μm thick layer of porous SiO_2 which is attached to the Al_2O_3 substrate, (2) a light-absorbing 0.5 μm thick layer of amorphous SiO_2, and (3) a top, 0.5 μm thick layer of SiO_2, to which oligomers having selected nucleotide sequences are attached ((2) in FIG. 5A; see (10), (11), and (12) in FIG. 2). The attached oligomers are 20-mer DNA oligonucleotides (20 nucleotides in length) that are covalently attached at their 3' ends to uncharged spacer groups, which spacer groups are covalently attached to the upper SiO_2 surfaces of the depot pads. 20-mer DNA oligonucleotides which are complementary to the attached oligomers are stored in the device by their being specifically hybridized to the attached oligomers by Watson-Crick base-pairing. The stored oligonucleotides have 5'-phosphate and 3'-OH termini, so that they can be ligated together.

The depot array is immersed in about 100 μl of solution containing 1 M NaCl, 5 mM EDTA, 0.1 M Tris-Cl, pH 8.0, 0.5% SDS.

As shown in FIG. 5A, the depot sites storing oligonucleotides a, b, and c, are each irradiated through the Al_2O_3 substrate with approximately 100 milliwatts of argon laser light (488 nm) ((9) in FIG. 5A) to melt double-stranded oligonucleotide complexes at the heated depots and release the desired single-stranded DNA oligonucleotides molecules into the solution ((5) in FIG. 5).

The 3' half of oligomer a and the 5' half of oligomer b are complementary, respectively, to the 3' and 5' halves of

oligomer c. Thus as shown in FIG. 5B, oligomer c hybridizes to the 3' end of oligomer a, and also to the 5' end of oligomer b, and it functions as a molecular splint by aligning the a and b oligomers end-to-end so that they can be covalently joined by ligase enzyme to produce a longer DNA molecule.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 16

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTTAACATA GGAATTTGAG GCAGTACGCA AAAA 34

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGCGTACTGC CTCAAATTCC TATGTTAAGA 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGCGTACTGC CTCAAATTCC TATGTT 26

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

NNBNNCGAGA CTGCACT 17

-continued

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGTGCAGTCT CGA 13

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTGCAGTCT CGT 13

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGTGCAGTCT CGG 13

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGTGCAGTCT CGC 13

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

NNNGTCTCN 9

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs

-continued

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

NNNNNGAGAC NNN 13

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGCGTACTGC CTCAAATTCC TATGTTNNNN NCGAGACTGC ACT 43

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGTGCAGTCT CGNTCTTAAC ATAGGAATTT GAGGCAGTAC GCAAAAA 47

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

NTCTTAACAT AGGAATTTGA GGCAGTACGC AAAAA 35

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGCGTACTGC CTCAAATTCC TATGTTN 27

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGTGCAGTCT CG 12

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

NNNNCGAGAC TGCACT 16

We claim:

1. A method for providing a set of oligomers comprising known subunit sequences comprising:

a) obtaining a device for storing and providing oligomers comprising a substrate that supports an array of oligomer depots;

wherein each depot comprises a delimited area or volume at which is attached a plurality of oligomers having a known subunit sequence, said oligomers being oligonucleotides and/or oligonucleotide analogs;

wherein the subunit sequence of the oligomers attached to at least one of said depots is different from the subunit sequence of the oligomers attached to a different depot of said array; and

wherein oligomers comprising known subunit sequences are stored at a plurality of depots of said array by being hybridized by Watson-Crick pairing to the oligomers attached at said depots to form double-stranded complexes, said stored oligomers also being oligonucleotides and/or oligonucleotide analogs;

b) locally heating one or more selected depots of the intact array to denature double-stranded complexes of said selected depots and release oligomers stored therein, without effecting significant denaturation of double-stranded complexes of the unselected depots of the array; and

c) collecting the oligomers released as a result of locally heating the selected depots.

2. The method of claim 1, wherein the oligomers attached to said depots and the oligomers that are hybridized to said attached oligomers are selected from the group consisting of DNA oligonucleotides, RNA oligonucleotides, DNA oligonucleotide analogs, and RNA oligonucleotide analogs.

3. The method of claim 1, wherein the oligomers attached to said depots and the oligomers that are hybridized to said attached oligomers are from 4 to 1000 subunits in length.

4. The method of claim 3, wherein the oligomers stored in the depots are about 8 to 30 subunits in length.

5. The method of claim 1, wherein the depot array comprises 2 to 10^7 depots.

6. The method of claim 5, wherein the depot array comprises 10^2 to 10^7 depots.

7. The method of claim 1, wherein the depots are supported by a rigid substrate.

8. The method of claim 1 wherein the depot sites range in diameter from about 1 micron to about 1 centimeter.

9. The method of claim 1 wherein the depot sites are thermally isolated from each other.

10. The method of claim 1 wherein said selected depots are heated by irradiation from a radiant energy source or by application of electric current to electronic heating elements.

11. The method of claim 10 wherein two or more of said selected depots are heated serially.

12. The method of claim 10 wherein two or more of said selected depots are heated at the same time.

13. The method of claim 1, further comprising:

d) allowing at least one of said oligomers from step c) to hybridize specifically to a complementary nucleotide sequence in a template nucleic acid, and contacting said hybridized oligomer with an enzyme with nucleic acid polymerase activity so that the hybridized oligomer is extended from its 3' end and a nucleic acid fragment complementary to a portion of the template nucleic acid is synthesized.

14. The method of claim 1, further comprising:

d) allowing at least one of said oligomers from step c) to hybridize specifically to a complementary nucleotide sequence in a nucleic acid template molecule comprising a first nucleic acid fragment to be amplified which is positioned on the 3' side of said complementary nucleotide sequence;

allowing at least one of said oligomers from step c) to hybridize specifically to a complementary nucleotide sequence in a nucleic acid template molecule comprising a second nucleic acid fragment to be amplified which is positioned on the 3' side of said complementary nucleotide sequence, and which is complementary to said first nucleic acid fragment to be amplified;

contacting said hybridized oligomers with an enzyme with nucleic acid polymerase activity so that the hybridized oligomers are extended from their 3' ends and nucleic acids comprising said nucleic fragments to be amplified are synthesized;

denaturing the resulting double-stranded nucleic acids, and repetitively carrying out said hybridization, polymerization, and denaturation steps to as to amplify said nucleic fragments to be amplified.

15. The method of claim 1, further comprising:

d) allowing at least one of said oligomers from step c) to hybridize specifically as a probe to a complementary nucleotide sequence in a target nucleic acid.

16. The method of claim 1, further comprising:

d) hybridizing at least two oligomer molecules from step c) end-to-end to adjacent, complementary nucleotide sequences in at least one splint nucleic acid, and

covalently joining the 3'-OH end of at least one of the hybridized oligomers to the 5'-phosphorylated end of an adjacent hybridized oligomer by an enzyme with ligase activity.

17. The method of claim 1, further comprising:

d) using said oligomers from step c) to determine a solution to a mathematical problem that is solvable by DNA-based computation.

* * * * *

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of	:	Staehler et al.
Application No.	:	10/579,769
Title	:	Highly Parallel Template-Based DNA Synthesizer
Art Unit	:	1637
Examiner	:	Thomas, David C.

Transmittal

Attached herewith are the following documents

- Third Party Submission Under 37 CFR 1.99
- U.S. Pat. No. 6,248,521
- U.S. Pat. No. 6,150,102
- Authorization to charge required fees
- Postcard receipt.